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ALTERATION OF KEY CYTOKINE LEVELS BY AFLATOXIN B<sub>1</sub> AND  
T-2 TOXIN IN MALE CD-1 MICE

by

Raviprakash R. Dugyala

A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Toxicology

Approved:

UTAH STATE UNIVERSITY  
Logan, Utah

1995

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## ABSTRACT

Differential Alterations of Key Cytokine Production by  
Aflatoxin B<sub>1</sub> and T-2 toxin in Male CD-1 Mice

by

Raviprakash R. Dugyala, Doctor of Philosophy  
Utah State University, 1995

Major Professor: Dr. Reed P. Warren  
Program: Toxicology

Aflatoxin B<sub>1</sub> and T-2 toxin are mycotoxins, which produce their immunotoxic effects by affecting nonspecific and acquired immunity in different species. The mechanisms of their immunotoxicity are still obscure. Cytokines are the key signaling molecules during the immune response. In this study, expression of macrophage-produced cytokines Interleukin-1 $\alpha$  (IL-1 $\alpha$ ), tumor necrosis factor (TNF), and IL-6, and lymphocyte-produced cytokines IL-2, interferon  $\gamma$  (IFN $\gamma$ ), and IL-3 was measured at the mRNA and protein levels, after *in vitro* activation with mitogens in AFB<sub>1</sub>- and T-2-toxin-exposed mice.

Significant changes in the organ weights, especially in the mice exposed to a high dose of T-2 toxin, and no effect in AFB<sub>1</sub>-exposed mice were observed.



ConA-induced production of IL-2, IFN $\gamma$ , and IL-3 mRNA and protein levels in AFB $_1$ -exposed mice showed a decrease in low dose groups (significant for IL-2 mRNA), but no change at other doses. However, in T-2-toxin-treated animals, there was a significant induction of IL-2 and IFN $\gamma$  mRNA in high and low doses and of IL-3 mRNA at the medium dose. The protein levels of IL-2 and IFN $\gamma$  did not follow the mRNA levels in high dose and the protein levels of IL-3 were significantly increased in medium and low doses.

LPS-induced IL-1 $\alpha$  and TNF mRNA and protein levels in AFB $_1$ -exposed mice were suppressed at the high dose while mRNA levels of both cytokines were increased significantly in the low and medium doses. Low and medium doses of AFB $_1$  also significantly decreased IL-1 $\alpha$  protein levels and the high dose decreased IL-6 protein. In T-2 toxin-treated mice, no significant difference in mRNA levels of these cytokines was observed but a general pattern of significant suppression of their protein levels (except IL-1 $\alpha$  at medium dose) showed that both toxins regulate the cytokine expression differently.

Based on the above discussed results and others, AFB $_1$  may alter cell-mediated immunity by affecting the communication between macrophages and T lymphocytes through inhibiting the macrophage-producing cytokines. T-2 toxin-induced immunosuppression may be due not only to the inhibition of

macrophage-producing cytokines, but also to the lack of effector cells to respond to the cytokines (IL-2, IFN $\gamma$ , and IL-3).

(153 pages)

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Raviprakash R. Dugyala

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## CHAPTER I

### INTRODUCTION AND LITERATURE REVIEW

Immunotoxicology is the discipline of toxicology devoted to the study of the immune system as a target for the toxicity of xenobiotics, drugs, and chemicals. This area is recently receiving a great deal of attention because a number of xenobiotics, drugs, and chemicals are proven to be immunomodulators. The immune system is very complex; it consists of a large number of different types of cells that are rapidly proliferating in a highly organized network. This network is very sensitive to several external factors. Any imbalance in the system may lead to immunosuppression, allergy, autoimmunity, or cancer. Cytokines or interleukins (ILs) are soluble factors produced by macrophages, T lymphocytes, and other cell types of the body when they are activated by specific antigens. They are the key signaling molecules between the immune cells and act in that microenvironment to help in the development of acquired immunity. Any modulation in the production of ILs will lead to either immunosuppression or immunostimulation. Results from the use of immunosuppressive drugs such as cyclosporin A and FK506, which are known to suppress the production of cytokines, suggest that mycotoxins, which are also immunomodulators, might cause immunotoxicity by interfering with the production of cytokines.

## CYTOKINES AND THEIR ROLE IN THE IMMUNE RESPONSE

Cytokines are soluble factors produced by macrophages, T lymphocytes, and other cell types of the body when they are activated by specific antigens. Macrophages produce cytokines such as IL-1 (IL-1 $\alpha$  and IL-1 $\beta$ ), IL-6, and tumor necrosis factor (TNF) (Akira *et al.*, 1990). There are two different subtypes of T-helper cells: T<sub>H</sub>1 cells that produce IL-2 and interferon  $\gamma$  (IFN $\gamma$ ) and T<sub>H</sub>2 cells that produce IL-4 and IL-10; IL-3 is produced by both cell types (Mosmann and Coffman, 1989). Cytokines play very important roles in promoting cell growth, differentiation, and functional activation of immune cells. These growth and differentiation factors act on different cell types by autocrine or paracrine activity to help clear the antigens in different ways. Current research indicates that cytokines are not restricted to one specific function but are multifunctional molecules with a variety of activities, some of which are overlapping with those of other cytokines. This kind of pleiotropy and redundancy of cytokines is exerted not only on the immune cells but also on many target cells and tissues, including even the nervous system (reviewed by Mizel, 1989).

Macrophages or monocytes are important in the regulation of humoral and cell-mediated immunity apart from their phagocytic and cytotoxic action against foreign invaders. When foreign antigens enter the body, they are taken and processed by macrophages and presented to T cells, thereby activating the T

cells. In this process, macrophages produce cytokines like IL-1 (IL-1 $\alpha$  and -1 $\beta$ ), IL-6, and TNF, and activated T lymphocytes (mainly T-helper cells) produce IL-2, IL-3, and IFN (reviewed by Mizel, 1989; Germain, 1994). These growth and differentiation factors act on different cell types by autocrine or paracrine pathways to make them more effective in clearing the antigens in different ways; these will be addressed briefly and individually.

**Interleukin-1.** Interleukin-1 (mainly derived from macrophages) is the term for the two polypeptides (IL-1 $\alpha$  and IL-1 $\beta$ ) that have a wide spectrum of inflammatory, metabolic, physiologic, hematopoietic, and immunologic properties. IL-1 is initially synthesized as a 33-kDa precursor, and subsequently processed to 13-17 kDa (Mizel, 1989). IL-1 participates in the mechanisms of T and B cell activation; its receptors are not expressed in the resting lymphocytes. The T cell activating property of IL-1 is measured by its ability to act in a co-stimulator assay with sub-optimal concentrations of antigens or mitogens (Dinarello, 1992). IL-1 amplifies T cell activation by inducing expression of IL-2 and IL-2R genes, particularly in conjunction with mitogens, antigens, calcium ionophores, or stimulators of protein kinase C (Simic and Stosic, 1985). IL-1, along with IL-4, also acts as a helper or co-factor during the activation process of B cells and Ig production. It also affects hematopoiesis by inducing the production of GM-CSF, C-CSF, M-CSF, and IL-3; it acts synergistically with CSFs and other cytokines on hematopoiesis (Bagby, 1989).

**Interleukin-6.** Interleukin-6 is a cytokine mainly produced by macrophages, with pleiotropic activities that play a central role in host defense. It is a glycoprotein with a molecular mass in the range of 20-30 kDa, depending on the cellular source and preparation (Akira *et al.*, 1993). IL-6 acts mainly on the late phase of the B cell differentiation pathway, consistent with the finding that interleukin-6 receptor is expressed on activated B cells but not resting B cells (Taga *et al.*, 1987). It acts on mitogen-activated B cells to induce IgM, IgG, and IgA production without stimulating B cell proliferation (Muraguchi *et al.*, 1988; Beagley *et al.*, 1989). IL-6 synergizes with IL-1 for T cell proliferation and cytotoxic T lymphocyte generation via induction of IL-2 (Houssiau and Van Snick, 1992). IL-6, along with IL-3, induces proliferation and differentiation of bone marrow progenitor stem cells (Suzuki *et al.*, 1989); it also enhances phagocytosis and expression of a number of macrophage differentiation antigens, including Mac-1 and Mac-3, major histocompatibility complex class I, nonspecific esterases, lysozyme, 2',5'-A-synthetase, and C-fms, indicating the functional differentiation into mature macrophages (Akira *et al.*, 1993).

**Tumor necrosis factor.** Tumor necrosis factor is largely produced by macrophages, and plays an important role in the defense against viral, bacterial, parasitic, immune, and autoimmune diseases. Tumor necrosis factor is synthesized as a precursor protein of 26 kDa, which is processed to a secreted 17 kDa (McGeeham *et al.*, 1994). The pleiotropic effects of TNF include cytostatic

and cytotoxic activity on malignant cells and cells infected by viruses or bacteria. It is also involved in the induction of cytotoxic T lymphocytes, enhancement of the killing activity of macrophages and natural killer (NK) cells, and activates the immune system by the generation of cytokines (IL-2, IL-7, IL-12) (Fiers, 1991).

**Interleukin-2.** Interleukin-2 plays a more pivotal role in initiating and potentiating the immune response than any other lymphokine secreted by T lymphocytes. It provides a critical signal necessary for both T cell proliferation and antibody secretion by B cells. As deduced from the cDNA sequence, the mature murine IL-2 contains 149 amino acids and molecular weights range from 15.5 to 31 kDa (Kashima *et al.*, 1985). The mechanism of IL-2 activation of T cells is the best understood among all known cytokines (Taniguchi and Minami, 1993). Interleukin-2 also helps in long-term growth and functional responsiveness of antigen-specific CTL clones (Gillis and Smith, 1977). It also induces the IL-2 receptor expression in T cells and acts on NK cells and monocytes (Smith and Cantrell, 1985; O'Garra *et al.*, 1988).

**Interleukin-3.** Interleukin-3 is a T lymphocyte-derived 28 kDa glycoprotein that contains 38% carbohydrate. The murine IL-3 gene encodes a protein of 166 amino acids. The first 26 amino acids encode a typical hydrophobic leader sequence required for secretion (Fung *et al.*, 1984). Interleukin-3 activates macrophages in different ways. Morphological changes and increased phagocytosis in IL-3-activated peritoneal macrophages were

reported (Crapper *et al.*, 1985). Interleukin-3 also induces Ia and the class II molecule LFA-1 expression in macrophages, thereby increasing the antigen presenting capacity to T-helper cells but not cytotoxicity (Frendl and Beller, 1990). Production of IL-1, IL-6, and TNF in macrophages is dramatically increased by the exposure of peritoneal macrophages to IL-3, showing that IL-3 is one of the key elements in macrophage activation (Frendl, 1992).

**Interferon  $\gamma$ :** Interferon  $\gamma$  is produced by T lymphocytes, including helper/suppressor phenotypes (Kasahara *et al.*, 1983). The protein contains 143 amino acids with a molecular weight ranging from 20 to 25 kDa. Although IFN $\gamma$  is best known for inducing anti-viral activity, it can also modulate the function of a number of cell types important in the immune response (Friedman and Vogel, 1983). IFN $\gamma$  plays a key role in the activation of T cells by stimulating increased expression of the IL-2R, which allows the continued proliferation of T cells in response to IL-2. In turn, the T cell response to IL-2 enhances the production of IFN $\gamma$  (Farrar *et al.*, 1986), thereby creating an interdependent network of T cell responses, which allows the continued production of soluble factors. This network increases tumor killing activity, phagocytosis, and induction of class II major histocompatibility antigen complex (Ia) in macrophages, and activation and growth enhancement of CTL-cells and NK-cells (Trincheiri and Perussia, 1985). IFN $\gamma$  is also known to induce oxygen-dependent, or respiratory burst-dependent microbicidal activity in the macrophage (Sasada and Johnson, 1980),

and oxygen-independent macrophage activity by enhancing indole amine 2, 3-dioxygenase (IDO) activity (Pfefferkorn, 1984).

The summary of the above discussed cytokine communication network during immune response is illustrated in Figure I-1.

#### REGULATION OF CYTOKINE GENE EXPRESSION

From the above discussion, it is clear that cytokines are very crucial signals for the development of the immune response. A single cytokine shows pleiotropism by acting on different targets, and different cytokines demonstrate redundancy by acting on the same target, showing similar effects. This is the main reason cytokines are regulated tightly at the transcriptional and translational levels: otherwise, the overproduction or underproduction of any particular cytokine would be very harmful to the body. For example, overproduction of TNF and IL-1 causes severe pathological symptoms such as inflammatory autoimmune diseases, one example of which is rheumatoid arthritis (Brennan and Feldmenn, 1992). Immunosuppressive effects of drugs such as cyclosporin A are mainly due to their effect on the down-regulation of IL-2 and other cytokines (Hess *et al.*, 1988). Regulation of cytokine expression may occur at the transcriptional, post-transcriptional, translational, or post-translational levels.



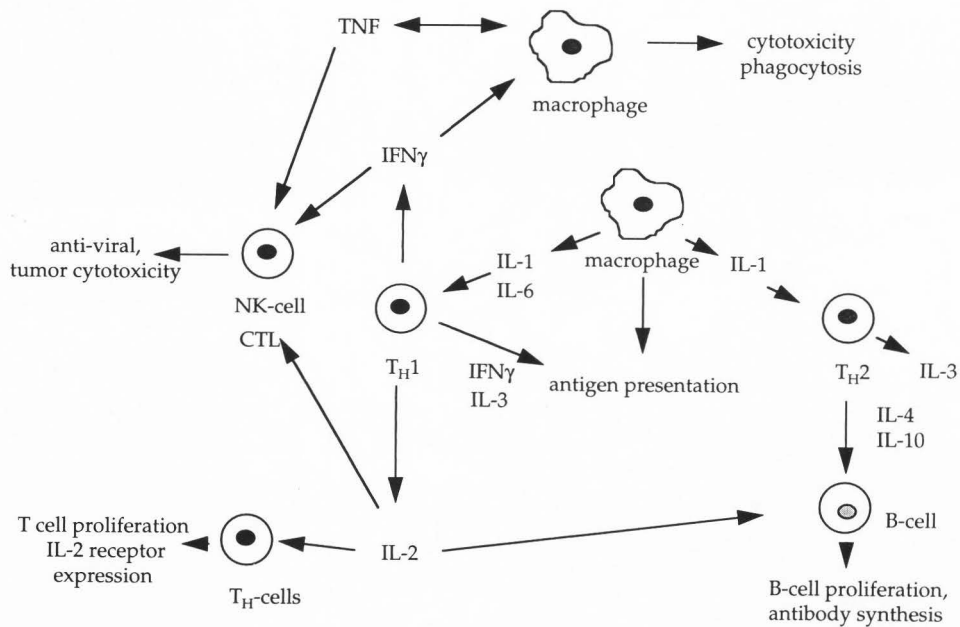


Fig. I-1. The cytokine communication network during immune response.

Cytokine genes, like many other eukaryotic genes, contain regulatory elements such as promoters, enhancers, and silencers. These elements are composed of discrete DNA sequences called *cis*-acting elements, which are binding sites for sequence-specific DNA binding proteins called *trans*-acting proteins. These *cis*-acting elements are generally directly upstream of the promoter and the two elements act in concert to achieve a fine degree of control over the transcriptional activity of the cytokine genes. *Trans*-acting factors such as NFAT (Nuclear Factor of Activated T cells), AP-1, AP-3, NF- $\kappa$ B (Nuclear Factor-kappa B), NF-IL6 and OBP (OCT-1 Binding Proteins) bind to the upstream regions of the promoters of cytokine genes and regulate the initiation of the transcription in a differential way (Riegel *et al.*, 1992; Chen-kiang *et al.*, 1993; Rao, 1994). Many immunosuppressive drugs such as cyclosporin A and FK506 are known to repress the induction of the transcription factors, thereby preventing the transcription of the cytokines (Emmel *et al.*, 1989; Brabletz *et al.*, 1991).

Post-translational mechanisms involving factors regulating the mRNA stability also play important roles in the gene regulation of cytokines. This is a very active area of research that remains unclear. Reiterated AUUUA pentamers (ARE) found within the 3'-untranslated regions (UTR) of IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, TNF, IFN $\gamma$ , G-CSF, and GM-CSF appear to function as *cis*-regulatory elements capable of regulating both the turnover and translation of these cytokines (Shaw and Karmen, 1986; Han *et al.*, 1990; Henics *et al.*, 1994;). This *cis*-regulatory

element appears to control the mRNA stability both positively and negatively by binding to different AU-rich sequence binding proteins (AUBP). In IL-2, AUBP bind directly to the AU-rich 3'-UTR and increase the stability of mRNA in tumor cells (Henics *et al.*, 1994). However, Cockfield *et al.* (1993) argued in their discussion that cycloheximide-induced superinduction of IFN $\gamma$  and TNF is due to its inhibition of synthesis of labile RNase proteins responsible for the degradation of mRNA targeting the AU-rich region. Further studies are ongoing to characterize different types of AUBP and their role in the regulation of turnover and translation of cytokine mRNAs.

Initiation of translation of regulatory proteins (growth factors, cytokines, etc.) is influenced by the different aspects of the mRNA structure: m7G cap, the primary sequence or context surrounding the AUG codon, leader length, and secondary structure both upstream and downstream of the AUG codon (Kozak, 1991). AU-rich 3'-UTR region in TNF is necessary for its efficient translation in endotoxin-treated AU-rich 3'-UTR constructs containing cell lines (Han *et al.*, 1990); this shows that secondary structural features of mRNA at the 3'-UTRs are very important for the efficient translation of these cytokines.

Production of interleukin-1 $\alpha$  and TNF is also regulated at the processing and secretion levels. IL-1 $\alpha$  and IL-1 $\beta$  do not possess a typical NH<sub>2</sub>-terminal hydrophobic secretory signal sequence to translocate directly into the cytoplasm via the classical exocytic secretory pathway (Giri *et al.*, 1985). As a consequence,

the newly synthesized IL-1 $\alpha$  precursor proteins are localized in the cytoplasm: There they are cleaved by the proteolytic enzyme calpain and undergo a conformational change that exposes a required secretory signal causing their secretion upon activation by a subsequent activation signal (Carruth *et al.*, 1991; Hazuda *et al.*, 1991; Siders *et al.*, 1993). Pentamidine, an aromatic diamine currently used to treat *Pneumocystis carinii*, specifically inhibits the cleavage of pro-IL-1 $\alpha$  and inhibits its secretion (Rosenthal *et al.*, 1991). A similar kind of inhibition of cleavage of pro-TNF into the secreted form is also observed with  $\Delta^9$ -tetrahydrocannabinol, a major psychoactive component of marijuana, in a mouse macrophage cell line (Fischer-Stenger *et al.*, 1993).

#### GENERAL TOXICOLOGY OF AFLATOXIN B<sub>1</sub> AND T-2 TOXIN

Aflatoxins (AFs) are produced by several strains of *aspergillus*, especially *A. flavus* and *A. Parasiticus*, that grow on food crops. The parent AFs, all of which occur naturally, are a group of structurally related compounds that possess a coumarin nucleus fused to a bisfuran moiety and either a pentanone, such as in AFB<sub>1</sub> and AFB<sub>2</sub>, or a six-membered lactone, as in the case of AFG<sub>1</sub> and AFG<sub>2</sub> (Figure I-2). Aflatoxin M<sub>1</sub> and AFM<sub>2</sub> are hydroxylated forms of AFB<sub>1</sub> and AFB<sub>2</sub>. Aflatoxin B<sub>2a</sub> and AFG<sub>2a</sub> are 8, 9-hydrated products of AFB<sub>1</sub> and AFG<sub>1</sub> (Coulombe, 1991; McLean and Dutton, 1995). Aflatoxin B<sub>1</sub> is usually found in the highest concentrations, followed by AFG<sub>1</sub>, AFB<sub>2</sub>, and AFG<sub>2</sub>. The order of acute

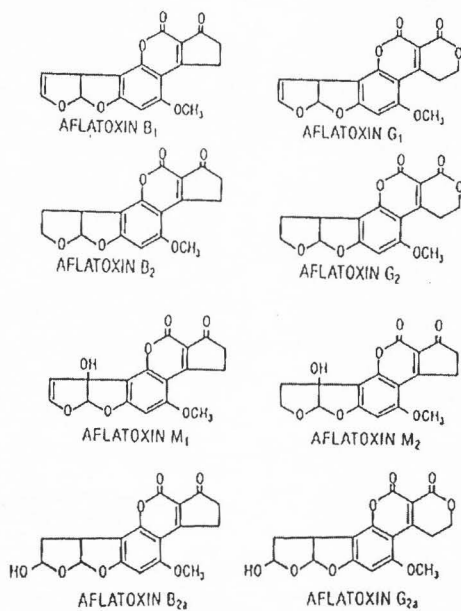


Fig. I-2. Structures of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, M<sub>1</sub>, M<sub>2</sub>, B<sub>2a</sub>, and G<sub>2a</sub>.

and chronic toxicity is  $AFB_1 > AFG_1 > AFB_2 > AFG_2$  (Wogan, 1966), reflecting the role played by epoxidation of the 8, 9-double bond and the greater potency associated with the cyclopentenone ring of the B series, when compared with the 6-membered lactone ring of the G series.

Aflatoxicosis caused by  $AFB_1$  and related toxins represents one of the most serious diseases of man, as well as poultry, livestock, and other lower animals such as rat and mouse. Acute aflatoxicosis in poultry causes hemorrhages in many tissues, decreases egg production, and causes anorexia, stunting, and hepatotoxicosis with icterus. Microscopically, evidence of hepatotoxicosis has included parenchymal degeneration with vacuolation, biliary hyperplasia, and necrosis followed by cirrhosis. Low concentrations have been reported to cause weakness and decreased resistance to disease, and have induced carcinogenesis in many species (Newberne and Butler, 1969; Edds, 1973; Mollenhauer *et al.*, 1989). One important property of  $AFB_1$  is its mutagenicity and carcinogenicity. Aflatoxin  $B_1$  causes hepatoma and other tumors in a variety of animal species (Robens and Richard, 1992). A number of epidemiological investigations have also implicated its role in the etiology of human liver cancer (Van Rensberg *et al.*, 1985; Yeh *et al.*, 1990). The chemical activity of  $AFB_1$  is thought to proceed via the formation of the 8, 9-epoxide that can react with DNA and RNA to form a covalent adduct at the N7 position of guanine residues (Croy *et al.*, 1978). The dihydrodiol form of  $AFB_1$ , which is the hydration product of  $AFB_1$ -8, 9, epoxide,

forms a schiffs base with amino groups of the bases (Hsieh, 1987). These interactions of AFB<sub>1</sub> with macromolecules will definitely affect their biosynthesis and increase possible mutations, which may induce cancer. Aflatoxin B<sub>1</sub> has been demonstrated to activate the *Ki-ras* gene in rat liver. In this regard, in the final stages of AFB<sub>1</sub>-induced rat liver hepatocellular carcinoma (HCC), two activating mutations in the codon 12 region of *Ki-ras* genes [GGT to GAT (McMahon *et al.*, 1987) and GGT to TGT (Sinha *et al.*, 1988)] have been identified. Soman and Wogan (1993) have confirmed the *Ki-ras* codon 12 GGT to GAT mutation in rat liver, suggesting the involvement of this genetic mutation in the development of AFB<sub>1</sub>-induced HCC in rats. Mutation in the third base of the codon 249 (G to T) in p53 tumor suppression gene has been shown in Chinese patients exposed to aflatoxin (Hsu *et al.*, 1991). However, there is a controversy involving these facts: Because these mutations do not correlate with the human HCC, there may be some other etiologic factors such as HBV infection also involved in the development of these liver cancers (Nose *et al.*, 1993).

Macromolecular syntheses such as DNA, RNA, and protein are also shown to be affected by AFB<sub>1</sub>. Covalent binding of AFB<sub>1</sub> to DNA leads to the modification of DNA template activity and/or inactivation of certain enzymes in DNA synthesis (Hsieh, 1987). RNA polymerase II activity and synthesis of rat liver RNA, especially nucleolar RNA synthesis, are inhibited by AFB<sub>1</sub> (Yu, 1977). RNA chain elongation and post-transcriptional processing of nuclear RNA are

also shown to be inhibited by the AFB<sub>1</sub> (Yu, 1981; Harley *et al.*, 1969). Inhibition of protein synthesis by AFB<sub>1</sub> may arise directly from inactivation of biosynthetic enzymes, or inhibition of translation, and/or interference with aminoacid transport (Hsieh, 1987). Disrupted membranes of endoplasmic reticulum due to the direct damage by AFB<sub>1</sub> may interfere with ribosome binding, and protein synthesis may be stopped (Terao and Ueno, 1978).

T-2 toxin is a secondary metabolite produced by *Fusarium tricinctum*, which grows on such crops as corn (Sharma and Salunkhe, 1991). It belongs to the family of mycotoxins called trichothecenes, and contains a common trichothecene skeleton with cyclopentane, cyclohexane, and a 6-membered oxyrane ring with four methyl groups. The skeleton contains an epoxide ring at C-12, 13 and a double bond at C-9, 10, characterized as 12, 13-epoxytrichothec-9-ene and are important for the trichothecene toxicity (Sharma and Kim, 1991). The numbering system is shown in Figure I-3. The trichothecenes are divided into simple and macrocyclic trichothecenes, depending on the presence of a macrocyclic ring linking at C-4 and C-5 with diesters or triesters. Simple trichothecenes are again divided into three types, namely type A, B, and C. Type A trichothecenes include T-2 toxin, neosolaniol, HT-2 toxin, and others. Type B trichothecenes, characterized by the presence of a ketone group in the C-8



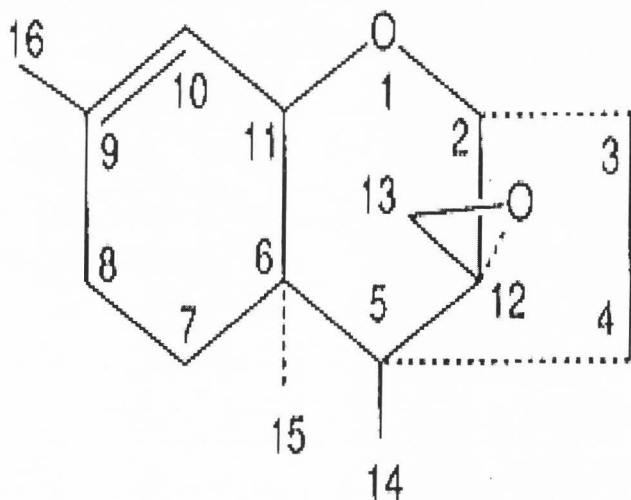


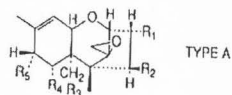
Fig. I-3. The numbering system of trichothecene.

position, include nivalenol, fusarium-X, and deoxynivalinol (Table I-1) (Sharma and Kim, 1991).

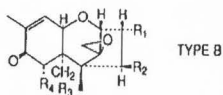
One of the best known human diseases associated with the trichothecenes in humans is alimentary toxic aleukia (Joffe, 1978). This disease may be divided into four stages clinically. Shortly after ingestion of contaminated food, symptoms such as burning sensation of mouth, tongue, throat, palate, esophagus, and stomach are evident due to the toxin's inflammatory action on the mucus membrane. Progressive leukopenia and granulopenia in the second stage will render the body susceptible to infections. Continuous intake of the toxin will cause petechial hemorrhages on the skin in the third stage followed by necrosis in tonsils and throat, causing angina in the final stage. Recently another human toxicosis in China was reported due to the ingestion of moldy rice contaminated with T-2 toxin (Wang *et al.*, 1993b). The main symptoms are nausea, dizziness, vomiting, chills, abdominal distention and pain, thoracic stuffiness, and diarrhea.

Gross and pathological changes are also observed in various large and laboratory animals treated with T-2 toxin. Swines treated with 0.6 to 5.4 mg (single dose) per kilogram body weight showed gross and microscopic changes (Pang *et al.*, 1987). Gross lesions include edema, congestion, hemorrhage of the lymph nodes, pancreas, gastrointestinal mucosa, subendocardium, etc. Microscopic lesions include degeneration and necrosis of lymphoid tissues and

TABLE I-1  
Structures of selected trichothecenes



Name	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>
T-2 toxin	OH	OAc	OAc	H	OCOCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>
HT-2 toxin	OH	OH	OAc	H	OCOCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>
Neosolaniol	OH	OAc	OAc	H	OH
Diacetoxyscipenol	OH	OAc	OAc	H	H
T-2 tetraol	OH	OH	OH	H	OH
Acetyl T-2 toxin	OAc	OAc	OAc	H	OCOCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>



Nivalenol	OH	OH	OH	OH
Pusarenon-X	OH	OAc	OH	OH
Diacetylivalenol	OH	OAc	OAc	OH
Deoxynivalenol	OH	H	OH	OH

epithelium of gastrointestinal mucosa, mild scattered necrosis of pancreatic cells, myocardium, bone marrow cells, adrenal cortical cells, and tubular epithelium of renal medulla. These findings suggested that T-2 toxin damages both rapidly dividing and nondividing cells. Oral exposure of male CD-1 mice with T-2 toxin (2.5 mg/kg) for 2-4 weeks also led to ulcerations, lymphocyte infiltration, epithelial proliferation, and hyperkeratinization of the forestomach (Taylor *et al.*, 1989).

T-2 toxin is cytotoxic to many cell types, causing damage to the tissues. Mice treated with T-2 toxin (0.30 mg/kg) had decreased bone marrow cellularity, suggesting that extremely small doses of T-2 toxin can produce a significant degree of bone marrow cytotoxicity (Faifer *et al.*, 1992). Hypocellularity of CD4+ and CD8+ T cells in thymus and T cells in spleen are observed in T-2-toxin-treated mice (Smith *et al.*, 1994), suggesting that T-2 toxin can be cytotoxic to specific subsets of cell types.

The mechanism of the toxicity of T-2 toxin is still not clear. T-2 toxin is a potent inhibitor of protein synthesis (McLaughlin, 1977): It selectively inhibits peptide bond formation in the late stage of translation initiation and early elongation (Cundliffe *et al.*, 1974; Smith *et al.*, 1975). The correlation observed among T-2 toxin binding to the 60S ribosome, cellular accumulation of the toxin, and the inhibition of protein synthesis (Middlebrook and Leatherman, 1989a, b) suggests that cytotoxic action of T-2 toxin may be due to the inhibition of protein

synthesis. DNA and protein synthesis are suppressed in bone marrow, spleen, and thymus in mice treated with T-2 toxin (0.75 mg/kg i.p.) for 8 and 20 hr (Rosenstein and Lafarge-Frayssinet, 1983), which shows that DNA synthesis inhibition is also a factor in the toxicity of T-2 toxin.

#### IMMUNOTOXICOLOGY OF AFLATOXIN B<sub>1</sub> AND T-2 TOXIN

Immunosuppressive effects of these mycotoxins have been explored in many animal species (reviewed by Sharma, 1993). Low-level aflatoxin intake has the potential to cause serious economic losses to the livestock producer through reduced productivity or through the impairment of resistance to infectious agents. Continuous treatment with AFB<sub>1</sub> in the diet (0.5 ppm) for 3 weeks reduced the acquired resistance to *Pasturella multocida* strain X-73 in turkey poults when these animals were immunized against the organism (Pier and Heddleston, 1970). Partially purified aflatoxin-fed pigs (760 mg) for 4 weeks increased the severity of *Erysipelothrix rhusiopathiae* infection (Cysewski *et al.*, 1978).

Impairment of phagocytosis was observed *in vitro* in heterophils when chickens were fed 0.625-10 ppm aflatoxin and presumed to be due to the inhibition of production of protein factors by the heterophils (Chang and Hamilton, 1979). Cell adherence and phagocytic potential of turkey peritoneal macrophages are decreased when AFB<sub>1</sub> (0.01, 0.1, 0.5, 1, and 5 mg) was added *in vitro* in the presence of a chicken microsomal mixed function oxidase system.

These results show that metabolized active forms of AFB<sub>1</sub> are responsible for the action (Neldon-Ortiz and Qureshi, 1991). Along with the peritoneal macrophages, macrophages resident in lungs and in the liver are also affected by AFB<sub>1</sub>. Alveolar and peritoneal macrophages from rats and mice treated with AFB<sub>1</sub> either by aerosol inhalation (3.17 mg/kg) for 60 min or intratracheal instillation (50-150 mg) showed suppression of phagocytosis (Jakab *et al.*, 1994). Aflatoxin B<sub>1</sub>, AFQ<sub>1</sub>, and M<sub>1</sub> (0.1-1 pg/ml) pretreated rat kupffer cells for 2-24 hr *in vitro* showed inhibition of phagocytosis, intracellular killing of *Candida albicans*, and anti-Herpes virus activity. But the AFB<sub>1</sub> is the more potent among the metabolites tested, suggesting that AFB<sub>1</sub> inhibits macrophage functions (Cusumano *et al.*, 1995).

Male CD-1 mice receiving 0.03-0.7 mg/kg AFB<sub>1</sub> orally every other day for 2 weeks showed decrease in T lymphocyte response (Reddy *et al.*, 1987). A decrease in [<sup>3</sup>H] thymidine uptake was seen when splenic cells were cultured with lipopolysaccharide (LPS), phytohemagglutinin (PHA), or poke weed mitogen (PWM). A decrease in T-dependent antibody production, no effect on T-independent antibody production, and a dose-related suppression of delayed-type hypersensitivity reaction to keyhole limpet hemocyanin were also observed in the above studies. In a similar study (Reddy and Sharma, 1989), where BALB/c mice are treated with similar concentration AFB<sub>1</sub> for 4 weeks, suppression of T-helper cells and T-suppressor cells, and NK-cell activity have

been observed. These studies clearly suggest that AFB<sub>1</sub> affects cell-mediated immunity even in very low doses.

Aflatoxin B<sub>1</sub> is considered a potent mutagen and carcinogen even in the immune cells; many studies have shown chromosomal aberrations due to AFB<sub>1</sub> exposure of immune cells. Significant aberrations in male Chinese hamster bone marrow cells are observed within 5 days of administration of AFB<sub>1</sub> (0.1-5 mg/kg) (Barta *et al.*, 1990). Graded doses of AFB<sub>1</sub> (1.09-17.4 mg/g embryo), applied on 18-day chick embryo, induced sister chromatid exchanges in the replicating DNA of T and B lymphocytes (Potchinsky and Bloom, 1993).

Aflatoxin B<sub>1</sub> is also known to affect the granulocyte-macrophage progenitor cell differentiation into formation of granulocyte and macrophage colonies *in vitro*. Bone marrow progenitor cells from AFB<sub>1</sub> (0.03-0.7 mg/kg)-treated mice showed a significant decreased capability to differentiate when cultured *in vitro* with the LPS-activated mouse serum (Dugyala *et al.*, 1994). Similar effects of inhibition of granulopoiesis by AFB<sub>1</sub> are also shown in rats (Cukrova *et al.*, 1992a), showing that AFB<sub>1</sub> may modulate immune functions by inhibiting the differentiation of progenitor cells into mature progenies.

Very few studies have looked into the alteration of cytokines by AFB<sub>1</sub>. Hatori *et al.* (1991) observed that C57Bl/6 mice treated with 0.03-0.75 mg/kg AFB<sub>1</sub> for 4 weeks showed a decrease in the production of IL-2 by splenocytes when activated with Con A *in vitro*. Increased production of IL-1 and IL-2 in

peritoneal macrophages and splenocytes, respectively, when activated with mitogens *in vitro*, is observed in rats treated with AFB<sub>1</sub> (i.p. 1 mg/kg, single dose) (Cukrova *et al.*, 1992b). Tumor necrosis factor production in alveolar macrophages is decreased in rats treated with AFB<sub>1</sub> (50-150 mg) intratracheal instillation for 3 days (Jakab *et al.*, 1994). In another *in vitro* study with the bovine monocytes, AFB<sub>1</sub> (10 mg/ml) pretreatment for 24 hr inhibited the IL-1 $\alpha$  mRNA levels and IL-1 secretion. The authors suggested that the inhibition is general since the concentration of AFB<sub>1</sub> used in this study also inhibited the constitutively expressed  $\beta$ -actin (Kurtz and Czuprynski, 1992). These results confirm that the cytokine modulatory role of AFB<sub>1</sub> and discrepancies found in the results in various studies may be due to the utilization of different experimental designs.

Immunosuppression is the common toxicological manifestation in humans as well as in various laboratory and food animal species following T-2 toxin exposure. Numerous studies have shown that the thymus is one of the main target organs of T-2 toxin. Other major organs of toxicity are bone marrow and spleen. Mice fed with T-2 toxin (20 ppm for 7-41 days) showed atrophy in thymus, Peyer's patches, and white pulp of spleen and bone marrow cells. Microscopically, decreased lymphocytes from thymic cortex, hypocellularity in bone marrow, and splenomegaly show that T-2 toxin is cytotoxic to the immune cells (Hayes *et al.*, 1980). Similar results were also observed in thymus and



spleens of CD-1 mice when treated with T-2 toxin (2.5 mg/kg for 2-4 weeks) (Taylor *et al.*, 1989). However, recent studies with the specific antibodies to the surface antigens of the different subsets of the lymphocytes showed that T-2 toxin does not show a general cytotoxicity in thymus and spleen but alters specific subtypes of T and B cells. T-2 toxin (1.75 mg/kg) exposure in mice showed an increase in CD8- CD4- (immature thymocytes) cells and a decrease in CD8+ CD4+ cells (Smith *et al.*, 1994), suggesting that T-2 toxin may affect thymic maturation. Mature T cells were also decreased in spleens in the same study, suggesting that cell-mediated immune functions are affected. T-2 toxin exposure (1.2 or 1.5 mg/kg) in mice selectively decreased the polymphoid cell population in the fetal liver (Holladay *et al.*, 1993; Holladay *et al.*, 1995). These studies provide support to the premise that lymphoid progenitor cells represent highly sensitive targets of T-2 toxin exposure.

Alteration of cell-mediated immune functions is also observed in T-2 toxin-exposed animals. Male CD-1 mice exposed to T-2 toxin (0.02-5.0 mg/kg) for 2-4 weeks showed suppression of PHA (T cell mitogen)-stimulation of splenic lymphocytes. Delayed type hypersensitivity is decreased even in the lower doses, suggesting that cell-mediated functions regulated by T cells are largely affected (Taylor *et al.*, 1985). Swiss mice treatment with T-2 toxin (i.p. with 0.75 mg/kg, 24 hr for 7 days) was also shown to prolong the period required for allograft rejection and decrease the T-dependent antibody production

(Rosenstein *et al.*, 1979). However, results from the data of various investigators should be analyzed cautiously before a conclusion can be reached due to their use of different experimental designs and treatment schedules. For example, mice receiving (i.p.) 1/2 LD 50 on day 0 and 1/4 LD 50 of crude T-2 toxin on day 1 showed a decrease in the PHA and LPS stimulation of splenocytes; and mice receiving (i. p.) 1/12 LD 50 of crude T-2 toxin for 15 days showed an increase in the PHA and LPS stimulation of splenocytes (Lafarge-Frayssinet *et al.*, 1979). These results show that T-2 toxin stimulates the lymphocyte functions in the low doses and suppresses them in the high doses of treatment.

Similar kinds of discrepancies were also observed in studies involving cell-mediated immunity to *Listeria monocytogenes* and T-2 toxin exposure. Preinoculation treatment with a single dose of T-2 toxin (p.o. 4 mg/kg) on day 2 or 4 prior to *Listeria* challenge significantly enhanced resistance and decreased mortality due to listeriosis by as much as 50%. In contrast, resistance was suppressed and mortality was increased by 50% in mice that were treated with toxin *after* the *Listeria* challenge (Corrier *et al.*, 1987).

T-2 toxin and other trichothecene effects on the macrophages were studied: T-2 toxin is cytotoxic to rat alveolar macrophages (AM) *in vitro* (Gerberick and Sorenson, 1983). Scanning electron microscope examination of AM treated with 0.1 mM T-2 toxin demonstrated detachment of pseudopodia, cellular blebbing, smoothing of membrane processes, and finally cell lysis. T-2

toxin also inhibited chemotaxis, chemiluminescence stimulated by bacteria, and phagocytosis of bacteria in rat leukocytes (Yarom *et al.*, 1984). T-2 toxin decreased both MHC class II (Ia) expression and antigen presentation by epidermal Langerhans cells in mice (Blaylock *et al.*, 1993). In another interesting study, enriched T lymphocytes from Fusarenon-X (a trichothecene)-treated mouse spleens showed no functional abnormality as they helped *in vitro* production of antibodies by PWM. However, there was a marked inhibition of antibody formation when the macrophages were added to the T lymphocytes, clearly showing that T-helper functions are inhibited by the Fusarenon-X-treated mice macrophages (Masuda *et al.*, 1982). The above discussed studies show that T-2 toxin and other trichothecenes affect cell-mediated immunity via inhibiting macrophage structure and functions.

Few studies have been conducted on the alteration of cytokines by T-2 toxin and other closely related trichothecenes. Spleen cells from mice treated for 4 consecutive days with 2 mg/kg T-2 toxin exhibited 4-fold higher levels of IL-2 than control mice but a 50% reduction in *in vitro* Con A activation (Holt *et al.*, 1988). A plausible explanation for the reduction of activity may be the lack of enough effector cells to respond to IL-2. Increased levels of IL-2, IL-4, and IL-5 are also observed in EL-4.IL-2 thymoma cells activated *in vitro* with Deoxynivalenol (trichothecene) (Dong *et al.*, 1994).

Stress response due to the T-2 toxin exposure may also play a role in T-2 immunomodulation. Endotoxemia is caused by the increased levels of endotoxin in the circulation, which is the result of T-2 toxin exposure in mice (Taylor *et al.*, 1989). A parallel increase of corticosterone due to the stimulation of hypothalamic-pituitary-adrenal axis, and the incidence of endotoxemia 24 hr after exposure to T-2, suggested that the stress response are due to systemic endotoxemia (Taylor *et al.*, 1989). In another study, brain biogenic monoamines, i.e., norepinephrine, serotonin, and 5-hydroxy-3-indoleacetic acid, were increased in T-2 toxin-exposed rats (Wang *et al.*, 1993a). The neuroendocrine system is known to affect the immune system (Fuchs and Sanders, 1994). Immunomodulatory effects of T-2 toxin may also be due to its indirect effects on the neuroendocrine system.

In summary, both toxins are found to be immunotoxic in various species by influencing both cell- and humoral-mediated functions affecting mainly T-helper cells and delayed type hypersensitivity functions. Macrophage functions like adherence, phagocytosis, and chemotaxis are also affected by both toxins. Many studies have shown the effects of xenobiotics on the immune system, but the mechanism of immunomodulation is still obscure. The main goal of this study is to investigate some of the molecular events that are involved in AFB<sub>1</sub> and T-2 toxin-induced immunotoxicity in male CD-1 mice. The working hypothesis is that these toxins may produce their immunotoxic effects via

interference with the synthesis of different cytokines in T lymphocytes and macrophages, thereby affecting the functions of T lymphocytes and macrophages and their interaction with other immune cells. Moreover, there is no single study demonstrating the inhibition of cytokines at transcriptional or translational levels by these toxins. Functions of macrophages are evaluated by their ability to produce IL-1 $\alpha$ , IL-6, and TNF and evaluation of T cell functions by measuring their ability to produce IL-2, IL-3, and IFN $\gamma$ . Both objectives are approached by quantifying the specific mRNA for each cytokine, employing Northern blot/RT-PCR techniques, and measuring the individual cytokine protein levels by sandwich ELISA, after *in vitro* induction with mitogens in control and toxin-treated animals.

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CHAPTER II

ALTERATION OF KEY CYTOKINES PRODUCED BY MITOGEN-  
ACTIVATED PERITONEAL MACROPHAGES AND  
SPLENOCYTES IN AFLATOXIN B<sub>1</sub>-TREATED  
MALE CD-1 MICE

ABSTRACT

Male CD-1 mice were used to test the *in vivo* effects of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) on the genetic expression of key cytokines that modulate activation and proliferation of splenic lymphocytes (IL-2, IFN $\gamma$ , and IL-3) and macrophages (IL-1 $\alpha$ , IL-6, and TNF) activated *in vitro* with LPS and Con A, respectively. Animals were treated with 0, 0.03, 0.145, or 0.7 mg AFB<sub>1</sub>/kg body weight orally every other day for 2 weeks. No significant effects of the toxin on the weights of liver, kidney, spleen, or thymus, or in red blood cell counts were noted, but white blood cell counts were significantly elevated at the low (0.03 mg/kg) dose. Cytokine mRNA levels were measured by RT-PCR or Northern blots and the secreted protein levels by ELISA. The low dose generally decreased both mRNA and protein levels of lymphocytic IL-2, IFN $\gamma$ , and IL-3, with IL-2 mRNA decreasing significantly from control levels ( $p \geq 0.05$ ). No significant differences in mRNA or protein levels between treated and untreated controls were found at the medium or high doses for the three cytokines. AFB<sub>1</sub> had a more marked

effect on macrophage-produced cytokines. While the mRNA levels increased significantly at the low (IL-1 $\alpha$ ) or medium dose (IL-6 and TNF), their corresponding protein levels were generally suppressed. The levels of IL-1 $\alpha$  secreted protein were significantly suppressed at all dosages, and those of IL-6 and TNF at high dose. Thus AFB<sub>1</sub> treatment appears to preferentially affect macrophage functions. These observations and how they relate to possible mechanisms of AFB<sub>1</sub> immunosuppression are discussed.

## INTRODUCTION

Aflatoxins are a group of mycotoxins produced by different strains of fungi such as *Aspergillus flavus* and *parasiticus*. Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) represents by far the most toxic of the aflatoxins (Coulombe, 1993). It is a potent carcinogen that causes hepatoma and other tumors in a variety of animal species (Robens and Richards, 1992). Hepatic and renal lesions in male broiler chickens, and hepatic lesions in turkey, ducklings, cattle, pig, sheep, rat, guinea-pig, mouse, dog, cat, rabbit, monkey, and humans show that AFB<sub>1</sub> affects almost all animal species (Mollenhauer *et al.*, 1989; Newborn and Buttler, 1969).

Immunotoxic potential of AFB<sub>1</sub> is known in many species, including laboratory and domestic animals (Sharma, 1993). Decreased lymphocyte response to different mitogens was observed in several studies. Aflatoxin B<sub>1</sub> is inhibitory to the phytohemagglutinin (PHA)-stimulated lymphocytes *in vitro* in

cattle and humans (Bodine *et al.*, 1984; Savel *et al.*, 1970). Male CD-1 mice receiving 0.03-0.7 mg AFB<sub>1</sub>/kg body weight orally every other day for 2 weeks showed a decreased T lymphocyte response to lipopolysaccharide (LPS), PHA, and poke weed mitogen (PWM), and T-dependent antibody production *in vitro* (Reddy *et al.*, 1987). Similar treatments in BALB/c mice for 4 weeks resulted in suppression of T-helper/suppressor cells along with the suppression of delayed type hypersensitivity and NK cell activity (Reddy and Sharma, 1989). These results strongly suggest that AFB<sub>1</sub> affects cell-mediated immunity.

Apart from the T lymphocytes, macrophages, which are also considered to be crucial in the immune response, are affected by the AFB<sub>1</sub>. Macrophages are involved in the first line of defense against infectious agents (Skamene and Gros, 1983); they process and present the antigen to the lymphocyte (Unanue, 1984) and secrete the cytokines that activate and stimulate proliferation of lymphocytes and other effector cells during the immune response (Akira *et al.*, 1990). Reduced phagocytosis and bactericidal abilities of heterophils from chicken were observed when fed with dietary aflatoxin (Chang and Hamilton, 1979). Inhibition of phagocytosis, intracellular killing of *Candida albicans*, and intrinsic anti-Herpes virus activity were observed in rat Kupffer cells when aflatoxins were added *in vitro* (Cusumano *et al.*, 1995). Aerosol and intratracheal instillation of AFB<sub>1</sub> inhibits phagocytosis in alveolar and peritoneal macrophages in rats and mice (Jakab *et al.*, 1994).



Although there is a large body of data demonstrating that AFB<sub>1</sub> affects immunity, little is known about the mechanisms of its immunotoxicology (Sharma, 1993). Suppression may occur when a toxin affects the phagocytic and/or antigen-processing capacity of the macrophages, the antigen recognition ability of the lymphocytes, the differentiation and proliferation of T and B lymphocytes, or the activation of immune effector mechanisms, including the production and release of cytotoxic lymphocytes, antibodies, and/or delayed hypersensitivity mediators. Since cytokines affect all the above-mentioned processes directly or indirectly, it is only logical that AFB<sub>1</sub> may produce its immunomodulatory effects through interference with the production of cytokines in macrophages and T lymphocytes.

Few studies have been done involving cytokine production and AFB<sub>1</sub> exposure. Hatori *et al.* (1991) observed a decrease in concanavalin A (Con A)-induced IL-2 production in mice treated with AFB<sub>1</sub>. Respiratory aflatoxicosis inhibits LPS-induced TNF $\alpha$  production in alveolar macrophages in rats (Jakab *et al.*, 1994). Interleukin-1 production was decreased in LPS/*Listeria monocytogenes*-induced bovine monocytes when pretreated with AFB<sub>1</sub> *in vitro* (Kurtz and Czuprynski, 1992). These studies, however, did not explain whether the analyzed cytokines are inhibited at the transcriptional or translational level by AFB<sub>1</sub>. In the present study, mRNA and protein levels for key cytokines were determined in peritoneal macrophages (IL-1 $\alpha$ , IL-6, and TNF) and splenic

lymphocytes (IL-2, IFN $\gamma$ , and IL-3) after exposure of CD-1 mice to various doses of AFB $_1$  *in vivo*. The cytokines were selected on the basis of their known importance in the regulation of acquired immunity (Paul and Sedar, 1994; Akira *et al.*, 1990).

## MATERIALS AND METHODS

**Animals.** Male CD-1 mice, 5 weeks old, were obtained from Charles River Laboratories (Wilmington, MA). Mice were acclimatized for 7 days in a temperature-controlled ( $22 \pm 1^\circ\text{C}$ , 50% relative humidity) and artificially illuminated room (12 hr light cycle) of an AAALAC-accredited animal care facility. Pelleted feed (toxin free) and fresh water were provided *ad libitum*.

**Aflatoxin B $_1$  exposure.** Aflatoxin B $_1$  was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in a corn oil:ethanol (95:5) vehicle just before administration. Two sets of mice were separated into four groups (control, low, medium, and high dose) and were dosed with 0, 0.03, 0.145, and 0.7 mg AFB $_1$ /kg body weight, respectively. The toxin was administered by oral gavage every other day for 2 weeks (seven doses). Body weights were recorded daily throughout the experimental period. Mice were euthanized by carbon dioxide 48 hr after they were given the final dose. From one set of animals, the livers, thymus, kidneys, and spleens were removed and weighed and the blood was collected in heparinized tubes for red and white blood cell counts. The spleens

were removed aseptically and used to isolate splenocytes. The second set of animals was used for isolation of macrophages by intraperitoneal lavage.

*Isolation and activation of splenic lymphocytes.* The aseptically collected spleens were maintained in cold complete RPMI [RPMI-1640 (Gibco, Grand Island, NY), 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-mercaptoethanol, 10% heat inactivated fetal calf serum (FCS) (Hyclone Lab., Logan, UT)]. Monocellular cell suspensions were prepared using a Stomacher lab blender (STOM 80, Tekmar Co., Cincinnati, OH). Red blood cells were removed with ACK lysing buffer (0.15M  $\text{NH}_4\text{Cl}$ , 1 mM  $\text{KHCO}_3$ , 0.1 mM  $\text{Na}_2\text{EDTA}$ , pH 7.4). The remaining cells were plated in Falcon 100-mm plates and incubated at 37°C for 1 hr to allow adherence of macrophages. The nonadherent cells were collected, counted by automatic blood cell counter (Coulter Electronic Inc., Hialeah, FL), adjusted to a concentration of  $2 \times 10^6/\text{ml}$ , activated with 5  $\mu\text{g}/\text{ml}$  Concanavalin A (Con A) (Sigma) in complete RPMI, and replated 15 - 20 ml/plate in 100-mm Falcon culture dishes for 12 or 48 hr.

*Isolation and activation of macrophages.* Animals to be used for isolation of macrophages were injected intraperitoneally with 3 ml 3% Brewers thioglycolate broth (BTG) (Gibco). On the fourth day post injection, peritoneal lavage was performed with 5 ml ice-cold complete RPMI + 10 U/ml heparin. Macrophages were counted in a hemacytometer, adjusted to  $10^6$  cells/ml in

complete RPMI medium, and 5-ml cell suspension were plated in 60-mm dishes. To activate the cells, lipopolysaccharide (LPS) (Sigma) was added to each plate at a concentration of 10  $\mu\text{g}/\text{ml}$  and cultured for 6 or 24 hr.

**Total RNA isolation and analysis.** Total RNA was extracted from lymphocytes after 12 hr activation with Con A and from macrophages after 6 hr activation with LPS. The lymphocytes were harvested by centrifugation and total RNA was extracted from the pellet with TRI REAGENT<sup>TM</sup> LS (Molecular Research Ctr., Cincinnati, OH) per manufacturer's protocol. RNA from macrophages was extracted by removing the medium and applying TRI reagent to the adherent cells on the plate following manufacturer's instructions. The purified RNAs were suspended in FORMAzol (Molecular Research) and stored at  $-20^{\circ}\text{C}$ .

Northern analysis of IL-1 $\alpha$ , IL-6, and TNF and control  $\beta$ -actin was done using antisense riboprobes labeled with  $\alpha$ - $^{32}\text{P}$ -UTP or  $\alpha$ - $^{32}\text{P}$ -CTP to a specific activity  $\sim 3 \times 10^8$  cpm. Antisense transcripts were generated using cloned cDNAs and the bacteriophage T3 or T7 RNA polymerase (Promega, Madison, WI), per manufacturer's instructions. Template cDNAs were as follows: for IL-1 $\alpha$ , ATCC clone #63106 in pBluescript SK+, cut with *Hind III* and transcribed with T3; for IL-6, 800 bp cDNA clone (*Pst I* ends) in pBluescript II KS+, cut with *Hind III* and transcribed with T7; for TNF, 1230 bp cDNA clone (*Eco R1* ends), in pBluescript

II.KS+, cut with *Hind III* and transcribed with T7. The latter two clones were obtained from Dr. A. D. Weinberg (University of California, San Diego, CA). Human  $\beta$ -actin used as control was from ATCC, clone #78554 cut with *Hind III*, transcribed with T3. All the clones were sequenced from both ends and found to conform to respective cDNA sequences in Genbank.

Ten micrograms of total RNA were fractionated through a 1.2% agarose gel containing 1.1% formaldehyde. The RNAs were transferred to MAGNA NT nylon membranes (Micron Separations Inc. Westboro, MA). The membranes were stained with 0.02% methylene blue and photographed, and the positions of 18S and 28S ribosomal RNA bands and RNA ladder size markers were noted on the membrane and later used for sizing and estimation of total RNA loaded per lane. Prehybridization (approx. 1 hr) and hybridization at 57°C (16 to 24 hr) were carried out in buffers containing 50% formamide and the membranes were washed under high stringency conditions per manufacturer's protocols. The blots were exposed from 6 hr to overnight to Kodak X-AR film at -70°C with Dupont Lightning Plus screens. The bands were quantitated by scanning their densities using ZERO-D scan software program (Stratagene Cloning Systems, LaJolla, CA). Total RNAs per lane were adjusted using  $\beta$ -actin standards and the percentage of cytokine RNA in mycotoxin-treated mice were calculated using untreated group values as 1.

IL-2, IL-3, and IFN $\gamma$  and  $\beta$ -actin were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) using Oligo 4.0-software-selected primer sets for each sequence as follows:

IL-2 (611 bp):	Sense= 5'-GCGCACCCACTTCAAGCTCC-3'
	Antisense= 5'-AGCCCTTGGGGCTTACAAAA-3'
IL-3 (567 bp):	Sense= 5'-CCCTTGGAGGACCCAGAAC-3';
	Antisense= 5'-GCCATGAGGAACATTCAGAC-3'.
IFN $\gamma$ (510 bp):	Sense= 5'-GTTCTGGGCTTCTCCTCCTG-3';
	Antisense= 5'-CGAATCAGCAGCGACTCCTT-3'.
$\beta$ -actin (265 bp):	Sense=AACACAGTGTTGTCTGGTGG-3';
	Antisense= 5'-ACGCAGCTCAGTAACAGTCC-3'

First-strand synthesis was carried out using 1  $\mu$ g of lymphocyte total RNA and 0.5  $\mu$ g oligo (dT)<sub>12-18</sub> (GibcoBRL), mixed in a 20- $\mu$ l total volume reaction containing 50 mM tris-HCl (pH8.3@RT), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM each dATP, dCTP, dGTP, and dTTP (Promega), and 200 U SuperScript II RNase H<sup>-</sup> RT (GibcoBRL) in a 50-min incubation at 42°C. After heating at 70°C for 15 min, the mixture was chilled in ice, and 2 units of RNaseH (Boehringer Mannheim, Indianapolis, IN) were added, followed by a 20-min incubation at 37°C. Twenty microliters of sterile double-distilled water was then added to the cDNA and 2  $\mu$ l of the diluted samples were used for each polymerase chain

reaction (PCR). The optimal  $\text{MgCl}_2$  and primer concentration in a PCR reaction were determined empirically for each cytokine as follows:  $\text{MgCl}_2$ : IL-2 and  $\beta$ -actin= 3mM; IL-3 and  $\text{IFN}\gamma$ = 2mM; Primer concentration: IL-2 and IL-3= 30 pmol;  $\text{IFN}\gamma$ = 20 pmol;  $\beta$ -actin= 10 pmol. Amplification was done in a TempCyclerII thermocycler (Coy Lab. Inc., Ann Arbor, MI) as follows: hot start at 95°C for 30 sec, followed by 25 cycles at 94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min.

The PCR products were run on 2% agarose gels, visualized by ethidium bromide, and quantitated by scanning photograph negatives as described for Northern blots.

**Cytokine quantitation.** Supernatants from macrophage cultures activated for 24 hr were used to quantitate IL-1 $\alpha$ , IL-6, and TNF. Supernatants from splenocyte cultures activated for 48 hr were used to quantitate IL-2, IL-3, and  $\text{IFN}\gamma$ . All supernatants were frozen and maintained at -70°C until assayed. All cytokines were quantitated by ELISA, using kits from either Genzyme Diagnostics, Cambridge MA (IL-1 $\alpha$ , IL-2, IL-6, TNF, and  $\text{IFN}\gamma$ ) or Endogen, Inc., Cambridge, MA (IL-3) per manufacturer's instructions.

**Statistical methods.** Data from these studies were analyzed by one-way analysis of variance (ANOVA) followed by the Fisher PLSD. Statistical calculations utilized Stat view<sup>TM</sup> SE +Graphics software from Abacus Concepts, Inc.

## RESULTS

Organ weight determination and cell counts revealed no effects of AFB<sub>1</sub> treatment on liver, kidney, spleen, or thymus weight or on red blood cell counts. The lowest dose (0.03 mg/kg body weight), however, caused a significant increase in the number of white blood cells when compared to the control groups. The medium dose, 0.145 mg/kg, also caused an increase in the number of white blood cells, but the increase was not significant. No marked changes in white blood cells were seen at the highest dose (0.7 mg/kg) (Table II-1).

The effect of AFB<sub>1</sub> on IL-2, IL-3, and IFN $\gamma$  gene expression was determined by RT-PCR analysis of total mRNA extracted from splenic lymphocytes of treated and untreated animals, after 12 hr activation with ConA *in vitro* (Figs. II-1-3). AFB<sub>1</sub> administered at the low dose of 0.03 mg AFB<sub>1</sub> per kg body weight decreased the level of all three mRNAs, with IL-2 registering a significant difference ( $p \leq 0.05$ ) from the control groups (Fig. II-1). No significant difference in IL-2, IL-3, and IFN $\gamma$  mRNA was found between control groups and animals treated at the medium (0.145 mg AFB<sub>1</sub>/kg body weight) or high (0.7 mg AFB<sub>1</sub>/kg body weight) dose levels. The levels of  $\beta$ -actin mRNA were similar in all the groups.

Interleukin-1 $\alpha$ , IL-6, and TNF gene expression in thioglycolate-broth-stimulated, AFB<sub>1</sub>-treated and control animals was determined by Northern blot analysis (Figs. II-4-6). Total mRNA was extracted from macrophages after 6 hr



TABLE II-1

Selected organ weights and blood cell counts of CD-1 mice exposed to AFB<sub>1</sub>  
for two weeks <sup>a</sup>

AFB <sub>1</sub> (mg/kg)	Organs (g/100 g body weight)				RBC	WBC
	Liver	Kidney	Spleen	Thymus	(x10 <sup>6</sup> /mm <sup>3</sup> )	(x10 <sup>6</sup> /mm <sup>3</sup> )
0	6.05 ± 0.23	1.69 ± 0.11	0.28 ± 0.02	0.28 ± 0.03	8.26 ± 0.43	8.20 ± 0.53
0.03	6.22 ± 0.40	1.62 ± 0.05	0.28 ± 0.02	0.28 ± 0.02	7.94 ± 0.12	12.36 ± 1.50*
0.145	6.15 ± 0.28	1.73 ± 0.12	0.34 ± 0.03	0.34 ± 0.02	7.98 ± 0.09	10.63 ± 0.49
0.7	5.73 ± 0.15	1.73 ± 0.06	0.27 ± 0.03	0.34 ± 0.03	7.94 ± 0.50	9.32 ± 1.25

<sup>a</sup> Values are given as mean ± S.E., n=5.

\*Significantly different from the control value (p < 0.05).

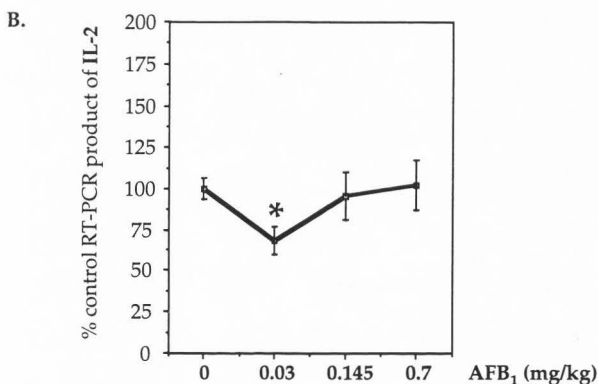
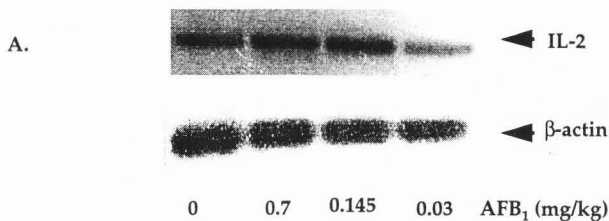


FIG. II-1. Effect of AFB<sub>1</sub> on the Con A-induced IL-2 expression. Total RNA (1  $\mu$ g) of the Con A-activated (5  $\mu$ g/2x10<sup>6</sup> cells for 12 hr) splenocytes from control and AFB<sub>1</sub>-treated mice were used to make cDNA and amplified by RT-PCR using murine IL-2 and murine  $\beta$ -actin primers(A). Band densities from five different animals were measured from the negatives of the ethidium bromide stained 2% agarose gels by Zero D-scan. Values of the total PCR product per lane were measured and equalized using  $\beta$ -actin standards, and the total IL-2 PCR product in the treated groups was calculated as percent of control values(B). Data points were mean  $\pm$  S. E. (n=5). (\*) Differences significant at  $p \leq 0.05$  level.

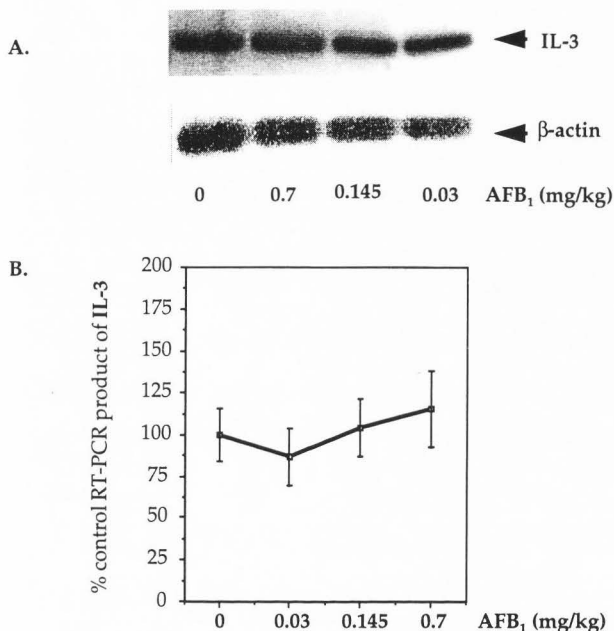


FIG. II-2. Effect of AFB<sub>1</sub> on the Con A-induced IL-3 expression. Total RNA (1  $\mu$ g) of the Con A-activated (5  $\mu$ g/2x10<sup>6</sup> cells for 12 hr) splenocytes from control and AFB<sub>1</sub>-treated mice were used to make cDNA and amplified by RT-PCR using murine IL-3 and murine  $\beta$ -actin primers (A). Band densities from five different animals were measured from the negatives of the ethidium bromide stained 2% agarose gels by Zero D-scan. Values of the total PCR product per lane were measured and equalized using  $\beta$ -actin standards, and the total IL-3 PCR product in the treated groups was calculated as percent of control values (B). Data points were mean  $\pm$  S. E. (n=5).

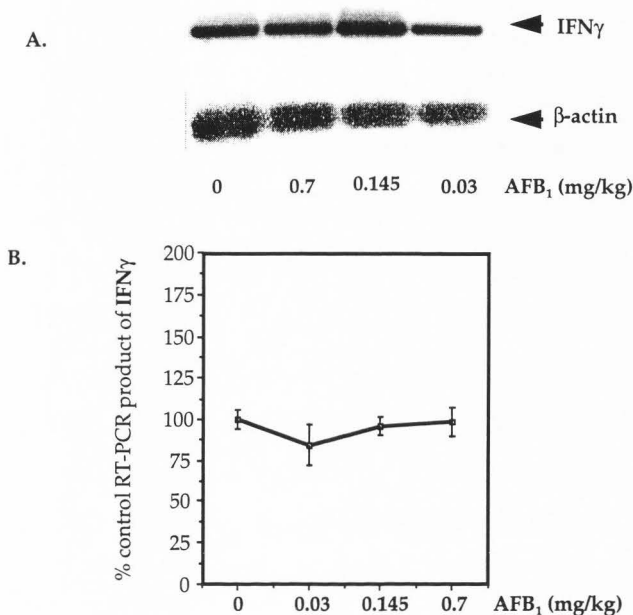


FIG. II-3. Effect of AFB<sub>1</sub> on the Con A-induced IFN $\gamma$  expression. Total RNA (1  $\mu$ g) of the Con A-activated (5  $\mu$ g/2 $\times$ 10<sup>6</sup> cells for 12 hr) splenocytes from control and AFB<sub>1</sub>-treated mice were used to make cDNA and amplified by RT-PCR using murine IFN $\gamma$  and murine  $\beta$ -actin primers (A). Band densities from five different animals were measured from the negatives of the ethidium bromide stained 2% agarose gels by Zero D-scan. Values of the total PCR product per lane were measured and equalized using  $\beta$ -actin standards, and the total IFN $\gamma$  PCR product in the treated groups was calculated as percent of control values (B). Data points were mean  $\pm$  S. E. (n=5).

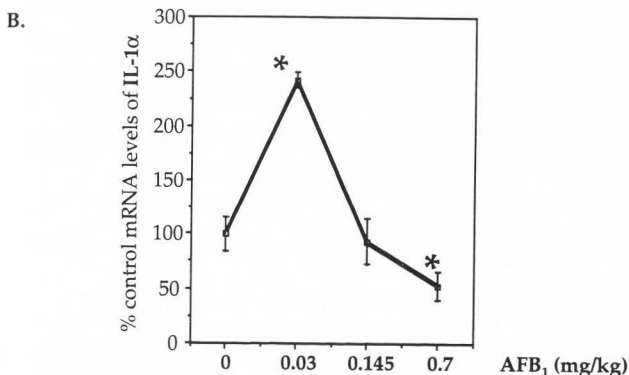
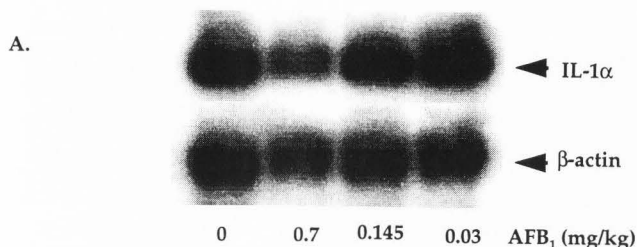


FIG. II-4. Effect of AFB<sub>1</sub> on the LPS -induced IL-1 $\alpha$  expression. Total RNA (10  $\mu$ g) of the LPS - activated (10  $\mu$ g/1x10<sup>6</sup> cells for 6 hr) peritoneal macrophages from control and AFB<sub>1</sub>- treated mice were analyzed by Northern blot using murine IL-1 $\alpha$  and human  $\beta$ -actin <sup>32</sup>P-labeled riboprobes (A). Band densities from three different animals were measured by Zero D-scan. Values of the total RNA per lane were measured and-equalized using  $\beta$ -actin standards, and the total IL-1 $\alpha$  mRNA in the treated groups was calculated as percent of control values(B). Data points were mean  $\pm$  S. E. (n=3). (\*) Differences significant at  $p \leq 0.05$  level.

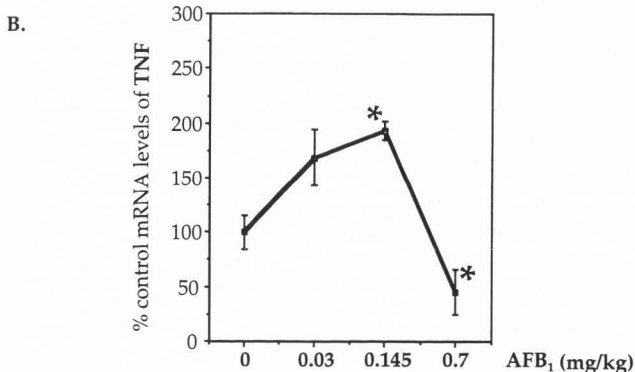
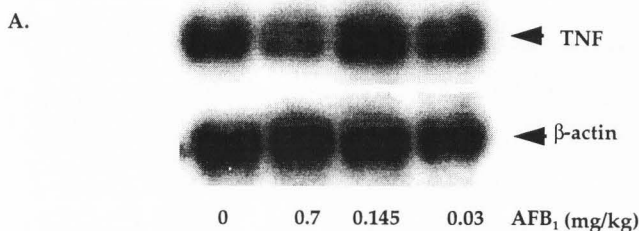


FIG. II-5. Effect of AFB<sub>1</sub> on the LPS-induced TNF expression. Total RNA (10  $\mu$ g) of the LPS-activated (10  $\mu$ g/1 $\times$ 10<sup>6</sup> cells for 6 hr) peritoneal macrophages from control and AFB<sub>1</sub>-treated mice were analyzed by Northern blot using murine TNF and human  $\beta$ -actin <sup>32</sup>P-labeled riboprobes (A). Band densities from three different animals were measured by Zero D-scan. Values of the total RNA per lane were measured and equalized using  $\beta$ -actin standards, and the total TNF mRNA in the treated groups was calculated as percent of control values (B). Data points were mean  $\pm$  S. E. (n=3). (\*) Differences significant at  $p \leq 0.05$  level.

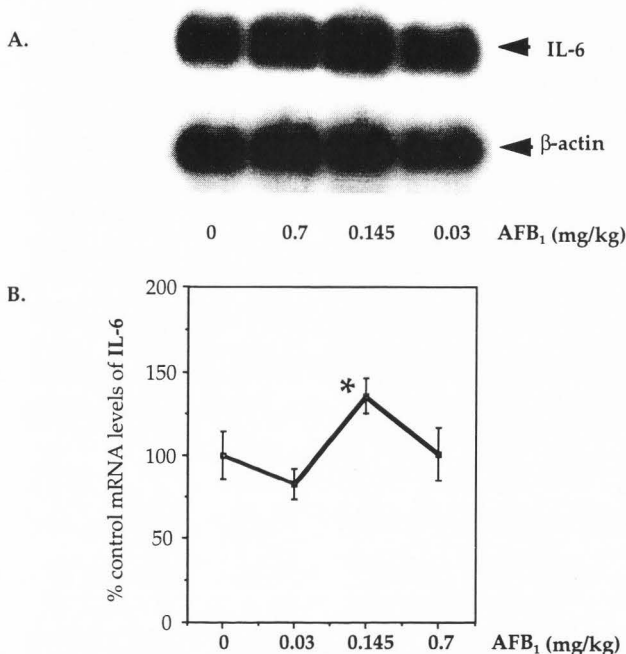


FIG. II-6. Effect of AFB<sub>1</sub> on the LPS-induced IL-6 expression. Total RNA (10  $\mu$ g) of the LPS-activated (10  $\mu$ g/ $1 \times 10^6$  cells for 6 hr) peritoneal macrophages from control and AFB<sub>1</sub>-treated mice were analyzed by Northern blot using murine IL-6 and human  $\beta$ -actin <sup>32</sup>P-labeled riboprobes (A). Band densities from three different animals were measured by Zero D-scan. Values of the total RNA per lane were measured and equalized using  $\beta$ -actin standards, and the total IL-6 mRNA in the treated groups was calculated as percent of control values (B). Data points were mean  $\pm$  S. E. (n=3). (\*) Differences significant at  $p \leq 0.05$  level.

LPS induction *in vitro*. The results show a significant increase in IL-1 $\alpha$  mRNA in animals treated with AFB<sub>1</sub> at low dose (Fig. II-4). The medium dose level groups show a significant increase in both TNF (Fig. II-5) and IL-6 (Fig. II-6) mRNA levels, while the high dose groups show a significant suppression of both IL-1 $\alpha$  and TNF (Figs. II-4, 5). No marked difference in the levels of  $\beta$ -actin was observed in any of the groups.

The effect of AFB<sub>1</sub> on the cytokine protein products was analyzed by ELISA. The supernatants of splenocyte cultures from treated and untreated animals were collected after 48 hr activation with Con A and used to quantitate IL-2, IL-3, and IFN $\gamma$ . Figs. II-7-9 show that for all three cytokines, AFB<sub>1</sub> treatment at all dose levels had no significant effect on the amount of protein secreted into the supernatant.

The pattern of protein production follows closely that observed for mRNA levels, i.e., a slight decrease of protein is observed at low dosage in all three cytokines, followed by a recovery to normal levels at medium and high dose levels. The only exception is IL-2, where a decrease in mRNA at low dosage is significant while the protein levels, although also suppressed, do not appear to be significantly different from the controls.

Similar ELISA analysis was performed for IL-1 $\alpha$ , IL-6, and TNF using the supernatant from macrophage cultures after LPS induction for 24 hr *in vitro*



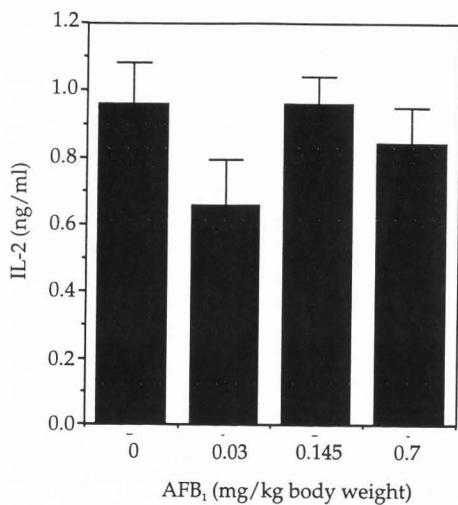


Fig. II-7. Effect of AFB<sub>1</sub> on IL-2 production. Splenocytes ( $2 \times 10^6$ /ml) from control and AFB<sub>1</sub>-treated mice were activated with 5  $\mu$ g/ml Con A for 48 hrs. Interleukin-2 in culture supernatants was detected by ELISA. Data were mean  $\pm$  S. E. (n=4).

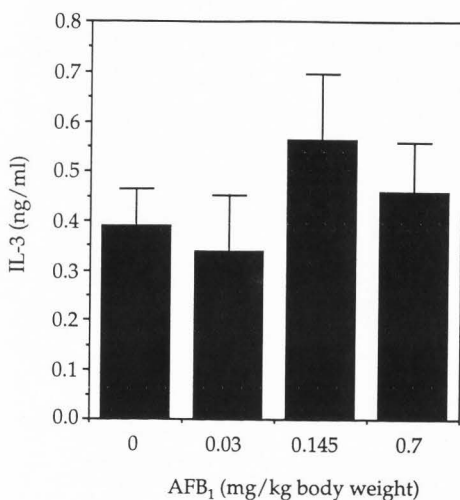


Fig. II-8. Effect of AFB<sub>1</sub> on IL-3 production. Splenocytes ( $2 \times 10^6$ /ml) from control and AFB<sub>1</sub>-treated mice were activated with 5  $\mu$ g/ml Con A for 48 hrs. Interleukin-3 in culture supernatants was detected by ELISA. Data were mean  $\pm$  S. E. (n=4).

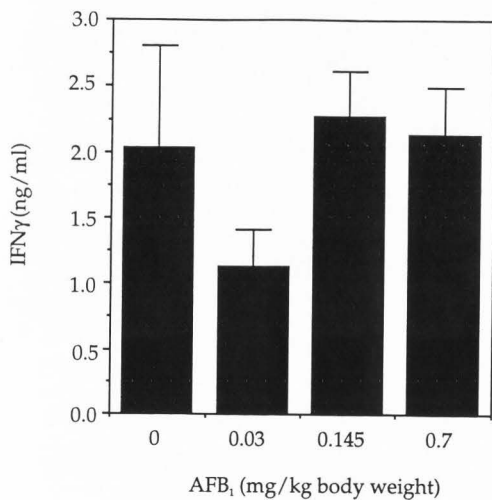


Fig. II-9. Effect of AFB<sub>1</sub> on IFN $\gamma$  production. Splenocytes ( $2 \times 10^6$ /ml) from control and AFB<sub>1</sub>-treated mice were activated with 5  $\mu$ g/ml Con A for 48 hrs. Interferon  $\gamma$  in culture supernatants was detected by ELISA. Data were mean  $\pm$  S. E. (n=4).

(Figs. II-10-12). A dramatic decrease in IL-1 $\alpha$  was observed after treatment at all dose levels of AFB<sub>1</sub>, and the suppression was significant when compared to the control groups ( $p \leq 0.05$ ) (Fig. II-10). A significant suppression of TNF and IL-6 was also observed, but only at the highest dose of AFB<sub>1</sub> (Figs. II-11, 12).

In contrast to IL-2, IFN $\gamma$ , and IL-3, where the levels of mRNA and protein seem to be closely correlated after all levels of AFB<sub>1</sub> treatment, IL-1 $\alpha$ , IL-6, and TNF mRNA and protein levels do not appear to be closely correlated. Messenger RNA appears to be superinduced at the low (IL-1 $\alpha$ ) or medium (IL-6 and TNF) dosage of AFB<sub>1</sub>, but this superinduction does not translate into increased amounts of cytokines secreted into the medium. In fact, there seems to be a generalized suppression of cytokine secretion at all treatment levels, with the effect being most significant for IL-1 $\alpha$ . Thus it appears that AFB<sub>1</sub> treatment preferentially affects macrophage functions, and, in particular, it decouples the close correlation usually observed between transcriptional and translational controls of IL-1 $\alpha$ , IL-6 and TNF production by these cells.

## DISCUSSION

This study used male CD-1 mice to describe the *in vivo* effects of AFB<sub>1</sub> on the genetic expression and production of several cytokines that modulate activation and proliferation of splenic lymphocytes and macrophages. Its underlying assumptions are that AFB<sub>1</sub> exerts its immunotoxic effects by

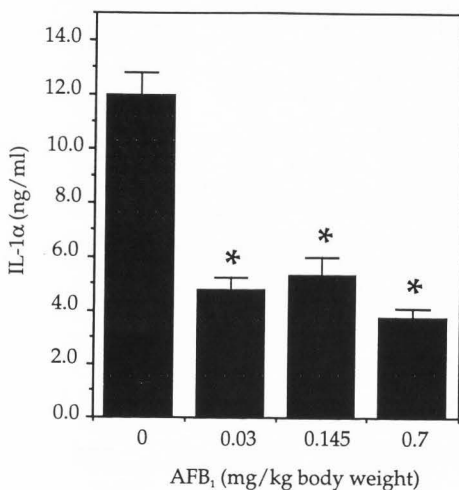


Fig. II-10. Effect of AFB<sub>1</sub> on IL-1 $\alpha$  production. BTG-stimulated peritoneal macrophages ( $1 \times 10^6$ /ml) from control and AFB<sub>1</sub>-treated mice were activated with 10  $\mu$ g/ml LPS for 24 hrs. Interleukin-1 $\alpha$  in culture supernatant was detected by ELISA. Data were mean  $\pm$  S. E. (n=3). (\*) Significant difference at 0.05 level between treatment and control groups.

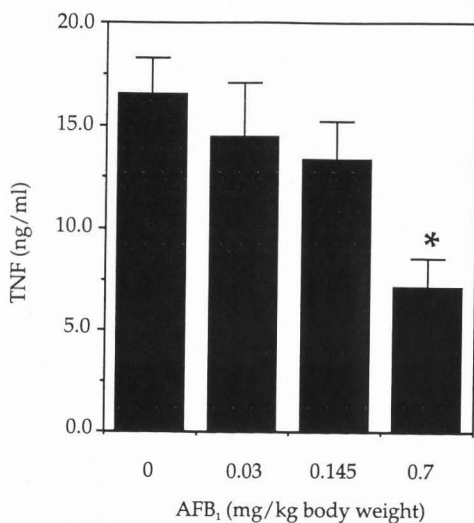


Fig. II-11. Effect of AFB<sub>1</sub> on TNF production. BTG-stimulated peritoneal macrophages ( $1 \times 10^6$ /ml) from control and AFB<sub>1</sub>-treated mice were activated with 10  $\mu$ g/ml LPS for 24 hrs. Tumor necrosis factor in culture supernatant was detected by ELISA. Data were mean  $\pm$  S. E. (n=3). (\*) Significant difference at 0.05 level between treatment and control groups.

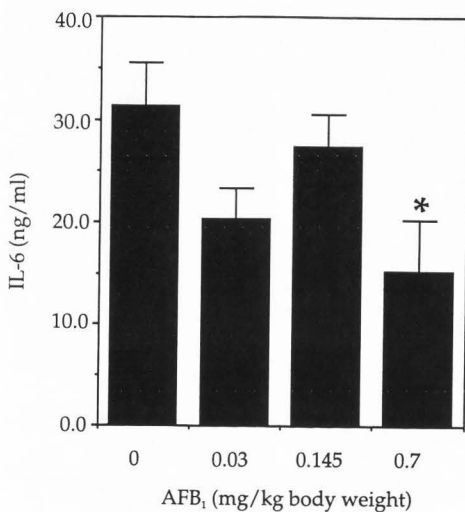


Fig. II-12. Effect of AFB<sub>1</sub> on IL-6 production. BTG-stimulated peritoneal macrophages ( $1 \times 10^6$ /ml) from control and AFB<sub>1</sub>-treated mice were activated with  $10 \mu\text{g/ml}$  LPS for 24 hrs. Interleukin-6 in culture supernatant was detected by ELISA. Data were mean  $\pm$  S. E. ( $n=3$ ). (\*) Significant difference at 0.05 level between treatment and control groups.

altering cytokine production in these cells and that analysis of the nature of those alterations will shed light on the molecular mechanisms involved in the toxin-induced immunosuppression.

The doses and duration of the AFB<sub>1</sub> exposure used in this study did not seem to be toxic as they did not affect the liver, kidney, thymus and spleen weights as described in previous reports (Reddy *et al.*, 1987; Reddy and Sharma, 1989; Dugyala *et al.*, 1994). There was no effect on the erythrocyte counts but there was a significant elevation in the leukocyte counts at low dose (0.03 mg AFB<sub>1</sub>/kg body weight), which was also observed in an earlier study (Dugyala *et al.*, 1994); this suggests that the doses of AFB<sub>1</sub> used in this study also have minor sub-clinical effects.

The levels of IL-2, IL-3, and IFN $\gamma$  protein secreted in the medium by Con A-treated lymphocytes corresponded in general to their respective mRNA levels but did not differ significantly from untreated control values, with the exception of IL-2 mRNA, which is suppressed significantly in the low-dose groups. Although not statistically significant, there was a general reduction of all three cytokine mRNA and protein levels at low dose. This is interesting in light of the fact that aflatoxin is known to affect cell-mediated immunity (i.e., suppression of delayed-type hypersensitivity, natural killer activity, and T-helper cell functions) in mice at low and repeated doses (Reddy *et al.*, 1987; Reddy and Sharma, 1989). Hatori *et al.* (1991) observed that C57B1/6 mice treated with similar doses used



in this study, showed a decrease in IL-2 production in the highest-dose-treated group and no effect in other AFB<sub>1</sub>-treated groups. The discrepancy may be due to their use of a 4-week exposure instead of 2 weeks used in the present study.

A number of factors may have caused the observed reduction in IL-2 and IFN $\gamma$  mRNA and protein levels at low doses of AFB<sub>1</sub>. CD4<sup>+</sup> T cells are subdivided into two distinct subsets based on the pattern of lymphocyte secretion; T<sub>H</sub>1 cells produce IL-2 and IFN $\gamma$  (and other cytokines) but not IL-4, IL-5, IL-6, and IL-10, and are involved in cell-mediated immune responses. T<sub>H</sub>2 cells produce IL-4, IL-5, IL-6, and IL-10, but not IL-2 and IFN $\gamma$ , and are involved in antibody-mediated immune responses (Mosmann and Coffman, 1989). IL-3 is produced by both types of cells. Emergence of the dominant subset of T-helper cells is dependent on the induction of specific cytokine profiles during the immune response. For example, macrophages, especially when infected by intracellular bacteria, are potent inducers of IL-12, directing the T-helper cell development toward T<sub>H</sub>1 responses (Hsieh *et al.*, 1993). In the absence of a source of high concentration of IL-12, production of IL-4 by subsets of CD4<sup>+</sup> T cells may prevent T<sub>H</sub>1 cell development and the production of cytokines (Gollob and Coffman, 1994; Yoshimato and Paul, 1994). Interleukin 10 produced by the T<sub>H</sub>2 cells, acting in conjunction with antigen presenting cells, also inhibits the T<sub>H</sub>1 production of IFN $\gamma$  and IL-2 (Fiorentino *et al.*, 1989). Cholera toxin, cyclosporin A, 8-Br-cAMP, and prostaglandin E2 are also known to preferentially inhibit T<sub>H</sub>1

cell-produced cytokines through their unique T cell receptor-associated signal transduction pathways (Gajewski *et al.*, 1990; Munoz *et al.*, 1990; Betz and Fox, 1991). Activation of RNA and protein synthesis in mouse splenocytes was observed in the low dose exposure to AFB<sub>1</sub> in BALB/c mice for 4 weeks (Reddy and Sharma, 1989). Activation of protein synthesis was also detected in CD-1 mice exposed for 2 weeks (Reddy *et al.*, 1987). Therefore, the low-dose inhibition of IL-2 and IFN $\gamma$  observed in this study may be due to activation of any of the above-discussed factors, which results in the suppression of T<sub>H</sub>1 cytokine products. Further studies are needed to explore these mechanisms of immunomodulation and the nature of T<sub>H</sub>1/T<sub>H</sub>2 cell subsets in CD4<sup>+</sup> T cell population during AFB<sub>1</sub> exposure.

The levels of IL-1 $\alpha$ , IL-6, and TNF protein secreted by LPS-stimulated macrophages varied significantly from the controls; IL-6 and TNF levels were reduced significantly at high dosages, and IL-1 $\alpha$  at all dosages administered. The fact that the mRNA levels did not correspond to those of the proteins at the low and medium dosages indicates a breakdown of the usual transcriptional regulation of these cytokines. In the high-dose-treated animals, both mRNA and protein decreased, significantly so for IL-1 $\alpha$  and TNF. The results show that AFB<sub>1</sub> suppresses IL-1 $\alpha$  gene expression and regulation transcriptionally in high dose groups and more strongly at the post-transcriptional level in all dose groups. Interleukin-1 $\alpha$  and IL-1 $\beta$  do not possess a typical NH<sub>2</sub>-terminal

hydrophobic secretory signal sequence responsible for translocation of the proteins directly into the endoplasmic reticulum via the classic exocytic secretory pathway (Giri *et al.*, 1985). As a consequence, the newly synthesized IL-1 $\alpha$  precursor protein is localized in the cytoplasm where it is cleaved by the proteolytic enzyme calpain; subsequently it undergoes a conformational change that exposes the required secretory signal to be secreted out upon an activation signal (Carruth *et al.*, 1991; Hazuda *et al.*, 1991; Siders *et al.*, 1993). Aflatoxin B<sub>1</sub> may affect any of the above discussed processes; it may inhibit the proteolytic enzyme or the subsequent events leading to the secretion of IL-1 $\alpha$ . Pentamidine, an aromatic diamidine currently used to treat *Pneumocystis carinii*, specifically inhibits the cleavage of pro-IL-1 $\alpha$  and its secretion (Rosenthal *et al.*, 1991).

Even though protein levels of IL-6 and TNF are not suppressed significantly, the fact that they do not correlate with the increased mRNA levels in the medium dose groups and the decreased protein levels in the high dose group shows that AFB<sub>1</sub> may affect some of the general translational machinery of the cell or its secretory pathway. Direct damage to the endoplasmic reticulum membranes, interference with the ribosome binding sites, interference with the ribosomal cycle, and inhibition of the release of newly synthesized proteins that may affect the ER-mediated protein synthesis are all alterations that have been observed during AFB<sub>1</sub> exposure (Terao and Ueno, 1978; Viviers and Schabort, 1985).

The significant changes observed in mRNA and protein levels for all three cytokines may be due to the differential regulation of factors responsible for transcription or mRNA stability by AFB<sub>1</sub>. Aflatoxin B<sub>1</sub> has the property of binding to the macromolecules via the formation of AFB<sub>1</sub>-8,9 epoxide and, to a lesser extent, its hydration product, the dihydrodiol form. The epoxide specifically makes an electrophilic attack on the N7 position of guanine in DNA and RNA (Croy *et al.*, 1978; Essigman *et al.*, 1980; Croy and Wogan, 1981a, b; Benasutti *et al.*, 1988), while the dihydrodiol forms a schiff base with amino groups of the bases (Hsieh, 1987). The inhibitory effects of AFB<sub>1</sub> may be on the DNA template activity and RNA polymerase II, an enzyme largely responsible for mRNA synthesis (Yu, 1977). AFB<sub>1</sub> also interferes with RNA chain elongation (Yu, 1981).

The elements in the 5' regulatory regions including glucocorticoid regulatory elements, NF $\kappa$ B, AP-1 binding sites, and NF-IL-6 have been shown to be involved in the transcriptional regulation, whereas AUUUA sequences in the 3'-untranslated region are the sites of cytosolic protein associations that stabilize/degrade the cytokine mRNA and regulate the stability and translational efficiency (Caput *et al.*, 1986; Shaw and Karmen, 1986; Brown and Beutler, 1990; Natsuka *et al.*, 1992; Henics *et al.*, 1994). Protein kinase C enhances the removal of poly (A) from TNF mRNA, thereby facilitating the early mRNA degradation (Lieberman *et al.*, 1992). Phorbol esters and calcium ionophores

have been shown to stabilize IL-2 mRNA via protein kinase C (PKC)-dependent phosphorylation as it is blocked by the inhibitors of PKC, such as staurosporine and K252a (Ohmura and Onoue, 1990). Moreover, the addition of an inhibitor of serine/threonine protein phosphatases (i.e., okadaic acid) resulted in increased instability of IL-2 mRNA, which further supports that protein phosphorylation plays an important role in the mRNA stability (Ohmura and Onoue, 1990). All these events require *de novo* protein synthesis, which can be altered by the AFB<sub>1</sub>. Immunosuppressant drugs such as FK506 and rapamycin inhibit mRNA stability of cytokines by a mechanism involving PKC-regulated AUUUA-specific binding proteins (Tocci *et al.*, 1989; Hanke *et al.*, 1992). There is evidence that AFB<sub>1</sub> binds to the cytoplasmic molecules destined for the nucleus (Ch'ih and Devlin, 1984; Ch'ih *et al.*, 1993). Moreover, AFB<sub>1</sub> preferentially binds to the physiologically active regions of the nucleolar chromatin of rat liver cells (Yu, 1983). A decrease of mRNA induction of IL-1 $\alpha$  and TNF and no effect on mRNA of IL-6 may be due to their differences in the time of expression and regulation (Akira *et al.*, 1990). Whether any of these processes are affected by AFB<sub>1</sub> in the immune cells needs to be further investigated.

Among the cytokines analyzed, IL-1 $\alpha$  seems to be more sensitive to AFB<sub>1</sub> at all dose levels tested than IL-6 and TNF; this shows that the mechanism of action of AFB<sub>1</sub> is specific. Interleukin 1 $\alpha$  is the most abundantly produced cytokine in mice; its level is above that of IL-1 $\beta$ . It plays a very important role in

immune regulation; together with IL-6 and TNF, it plays a pivotal role in cell-mediated immunity, as well as in the regulation of inflammation and hematopoiesis (Rosenthal *et al.*, 1991; Dinarello, 1992). Interleukin-1 amplifies T cell activation by inducing IL-2 and IL-2R gene expression, particularly in conjunction with antigens and mitogens (Dinarello, 1992). The activation of mature T cells by IL-1 is dramatically increased in combination with IL-6, which shows that the two cytokines act synergistically (Dinarello, 1992). Tumor necrosis factor also plays a role in the development of cell-mediated immunity by inducing the cytotoxic T lymphocytes and generating IL-2 and IL-12 (Fiers, 1991). Based on the present investigation, AFB<sub>1</sub> seems to affect cell-mediated immunity mainly by affecting the macrophage-producing cytokines. The results would be an inhibition of the signals activating T<sub>H</sub>1 cells to respond to foreign antigen. It does not seem to have much effect on T<sub>H</sub>1 cytokine production in spite of the slight decrease in production of Con A-induced cytokines by splenocytes in the low-dose-treated groups.

The observations strengthen the conclusion that AFB<sub>1</sub> main effects are on the macrophages. Various studies have shown that aflatoxin inhibits macrophage functions such as phagocytic, chemotactic, microbicidal, and adherence activity (Chang and Hamilton, 1979; Neldon-Ortiz and Qureshi, 1991; Jakab *et al.*, 1994; Cusumano *et al.*, 1995). These impaired functions of macrophages will definitely affect the first line of defense against infectious

diseases (Skamene and Gros, 1983). More importantly, macrophages take up and process foreign substances and present the derived antigens to lymphocytes, as well as secrete cytokines, which are important for the development of acquired immunity (Unanue, 1984). By adding the present results to other studies, the conclusion that AFB<sub>1</sub> mainly affects cell-mediated immunity via inhibition of macrophage functions is warranted.

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CHAPTER III

ALTERATION OF KEY CYTOKINES PRODUCED BY MITOGEN-  
ACTIVATED PERITONEAL MACROPHAGES AND  
SPLENOCYTES IN T-2 TOXIN-TREATED  
MALE CD-1 MICE

ABSTRACT

Male CD-1 mice were used to study the effect of *in vivo* exposure to T-2 toxin on the alteration of IL-1 $\alpha$ , TNF, and IL-6 cytokines in LPS-stimulated peritoneal macrophages, and IL-2, IL-3, and IFN $\gamma$  cytokines in Con A-stimulated splenocytes. Mice received 0, 0.1, 0.5, and 2.5 mg T-2 toxin/kg body weight orally for 2 weeks on alternate days. Northern blot analysis of IL-1 $\alpha$ , TNF, and IL-6 mRNA from activated peritoneal macrophages showed no significant differences between control and treated groups. ELISA measurements of secreted protein demonstrated suppression of these cytokines in all groups when compared to the controls. Thus T-2 toxin appears to affect the translational or post-translational regulation of cytokines from peritoneal macrophages. IL-2, IL-3, and IFN $\gamma$  mRNA levels from Con A-activated splenocytes were higher in all treated groups; the increases were significant for IL-2 and IFN $\gamma$  in the groups receiving low (0.1 mg/kg) and high (2.5 mg/kg) doses of T-2 toxin, and for IL-3 in the group receiving a medium (0.5 mg/kg) dose of this toxin ( $p \leq 0.05$ ). The



data indicate that T-2 toxin at low or medium doses induces transcription or increases mRNA stability of IL-2, IFN $\gamma$ , and IL-3. The protein levels of all three cytokines were also increased significantly; for IL-2 and IFN $\gamma$  at the low dosage, and for IL-3 at low and medium dosages, indicating that T-2 toxin also increases translational/post-translational efficiency of all three cytokines. The possible mechanisms involved in the immunosuppressive effects of T-2 toxin are discussed relative to these findings.

## INTRODUCTION

T-2 toxin [12, 13-epoxytrichothec-9-ene-3, 4, 8, 15-tetrol 4, 15-diacetoxy-8-(3-methyl-butanoate)] is one of the many trichothecene mycotoxins produced by several genera of fungi, particularly the *Fusarium* species. It causes severe human and animal diseases such as alimentary toxic aleukia and stachybotriotoxicosis, affecting the mucosa and the immune system. Leukopenia, anemia, and bone marrow aplasia are the most common pathological symptoms of these diseases (Sharma and Kim, 1991). T-2 toxin has systemic effects on nearly every major organ system, including cardiovascular, central nervous system, bone marrow, liver, and the gastrointestinal tract in animals and humans (Ueno *et al.*, 1973; Weaver *et al.*, 1978; Hayes and Schiefer, 1980; Hayes *et al.*, 1980; Lusky and Mor, 1981; Ueno, 1983; Joffe, 1986; Pang *et al.*, 1987; Dugyala *et al.*, 1991).

The immunotoxic potential of T-2 toxin in different animals has been reviewed and found to selectively affect various subpopulations of immune cells such as lymphocytes and macrophages (Sharma, 1993). Decrease of cytotoxicity, phagocytosis and chemotaxis were observed in human polymorphonuclear cells and rat alveolar macrophages when treated with T-2 toxin (Gerberick and Sorenson, 1983; Yarom *et al.*, 1984). CD-1 mice exposed for 2 or 4 weeks to T-2 toxin (0.02-5.0 mg/kg body weight) showed a suppression of thymus-derived T cell mediated function and delayed-type hypersensitivity but normal T cell dependent antibody production and antibody-producing cells (Taylor *et al.*, 1985). These data indicate that T-2 toxin has a specific immunomodulatory effect in mice. Some of the immunomodulatory effects of T-2 toxin are indirect, acting via the hypothalamic-pituitary-adrenal (HPA) axis in mice (Taylor *et al.*, 1989; Taylor *et al.*, 1991). T-2 toxin administered to pregnant mice has been found to specifically decrease prolymphoid cell populations in fetal thymus, liver and bone marrow cells (Holladay *et al.*, 1993; Holladay *et al.*, 1995). T-2 toxin has also been reported to lower lymphoid DNA and protein synthesis (Rosenstein and Lafarge-Frayssinet, 1983), and antibody synthesis (Rosenstein *et al.*, 1979). In experimental models, T-2 toxin has also been reported to cause decreased resistance to mycobacterial infection (Kanai and Kondo, 1984), increased mortality after challenge with *Salmonella* species (Tai and Pestka, 1990), increased susceptibility to herpes simplex virus (Friend *et al.*, 1983), and increased

mortality in listeriosis (Corrier *et al.*, 1987). While it is known that T-2 toxin inhibits cellular protein, DNA, and RNA synthesis (Corrier and Ziprin, 1986; Mann *et al.*, 1983; Lafarge-Frayssinet *et al.*, 1981; Gyongyossy-Issa and Kachatourians, 1985; Gyongyossy-Issa *et al.*, 1986; Deloach *et al.*, 1989), little is known about the mechanism involved in the immunomodulation.

Understanding the mechanism of immunosuppression of T-2 toxin is very difficult due to its complex effects on the immune response *in vivo*, which are still obscure. Suppression may occur when a toxin affects the phagocytic and/or antigen-processing capacity of the macrophages, the antigen recognition ability of the lymphocytes, the differentiation and proliferation of T and B lymphocytes, or the activation of immune effector mechanisms, including the production and release of cytotoxic lymphocytes, antibodies, and/or delayed hypersensitivity mediators. Since cytokines affect all the above-mentioned processes directly or indirectly, it is only logical that T-2 toxin may produce its immunomodulatory effects through interference with the production of cytokines in macrophages and T lymphocytes. Few studies have investigated cytokine (IL-2) production and T-2 toxin exposure (Holt *et al.*, 1988b). In the present study, mRNA and protein levels for several key cytokines were determined in macrophages (IL-1 $\alpha$ , IL-6, and TNF) and splenic lymphocytes (IL-2, IFN $\gamma$ , and IL-3) after exposure of CD-1 mice to various doses of T-2 toxin *in vivo*. The cytokines were selected on

the basis of their known importance in the regulation of acquired immunity (Akira *et al.*, 1990; Paul and Sedar, 1994).

## MATERIALS AND METHODS

**Animals.** Male CD-1 mice, 5 weeks old, were obtained from Charles River Laboratories (Wilmington, MA). Mice were acclimatized for 7 days in a temperature-controlled ( $22 \pm 1^\circ\text{C}$ , 50% relative humidity) and artificially illuminated room (12 hr light cycle) of an AAALAC-accredited animal care facility. Pelleted feed (toxin free) and fresh water were provided ad libitum.

**T-2 toxin exposure.** T-2 toxin was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in a corn oil:ethanol (95:5) vehicle just before administration. Two sets of mice were separated into four groups (control, low, medium, and high dose) and were dosed with 0, 0.1, 0.5, or 2.5 mg T-2 toxin/kg body weight, respectively. T-2 toxin was administered by oral gavage every other day for 2 weeks (seven doses). Body weights were recorded daily throughout the experimental period. Mice were euthanized by carbon dioxide after 48 hr of the final dose. From one set of animals, the livers, thymus, kidneys, and spleens were removed and weighed and the blood was collected in heparinized tubes for red and white blood cell counts. The spleens were removed aseptically and used as a source of splenocytes. Macrophages were isolated from the second set of animals by intraperitoneal lavage.

*Isolation and activation of splenic lymphocytes.* The aseptically collected spleens were maintained in cold complete RPMI [RPMI-1640 (Gibco, Grand Island, NY), 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-mercaptoethanol, 10% heat inactivated fetal calf serum (FCS) (Hyclone Lab., Logan, UT)]. Monocellular cell suspensions were prepared using a Stomacher lab blender (STOM 80, Tekmar Co., Cincinnati, OH). Red blood cells were removed with ACK lysing buffer (0.15 M  $\text{NH}_4\text{Cl}$ , 1 mM  $\text{KHCO}_3$ , 0.1 mM  $\text{Na}_2\text{EDTA}$ , pH 7.4). The remaining cells were plated in Falcon 100 mm plates and incubated at 37°C for 1 hr to allow adherence of macrophages. The nonadherent cells were collected, counted by automatic blood cell counter (Coulter Electronic Inc., Hialeah, FL), adjusted to a concentration of  $2 \times 10^6$ /ml, activated with 5 mg/ml concanavalin A (Con A) (Sigma) in complete RPMI, and replated 15 - 20 ml/plate in 100-mm Falcon culture dishes for 12 or 48 hr.

*Isolation and activation of macrophages.* For macrophages, animals were injected intraperitoneally with 3 ml of 3% Brewers thioglycolate broth (BTG) (Gibco). On the fourth day post injection, peritoneal lavage was performed with 5 ml ice-cold complete RPMI + 10 U/ml heparin. Macrophages were counted in a hemocytometer, adjusted to  $10^6$  cells/ml in complete RPMI medium, and 5 ml of the cell suspension were plated in 60-mm dishes. To

activate the cells, lipopolysaccharide (LPS) (Sigma) was added to each plate at a concentration of 10 mg/ml and cultured for 6 or 24 hr.

*Total RNA isolation and analysis.* Total RNA was extracted from lymphocytes after 12 hr activation with Con A and from macrophages after 6 hr activation with LPS. The lymphocytes were harvested by centrifugation and total RNA was extracted from the pellet with TRI REAGENT™ LS (Molecular Research Ctr., Cincinnati, OH) per manufacturer's protocol. RNA from macrophages was extracted by removing the medium and applying TRI reagent LS to the adherent cells on the plate following manufacturer's instructions. The purified RNAs were suspended in FORMAzol (Molecular Research) and stored at -20°C.

Northern analysis of IL-1 $\alpha$ , IL-6, and TNF and control  $\beta$ -actin was done using antisense riboprobes labeled with  $^{32}\text{P}$ -UTP or  $^{32}\text{P}$ -CTP to a specific activity  $\geq 3 \times 10^8$  cpm. Antisense transcripts were generated using cloned cDNAs and the bacteriophage T3 or T7 RNA polymerase (Promega, Madison, WI), per manufacturer's instructions. Template cDNAs were as follows: for IL-1, ATCC clone #63106 in pBluescript SK+, cut with *Hind* III, transcribed with T3; for IL-6, 800 bp cDNA clone (*Pst* I ends) in pBluescript II KS+, cut with *Hind* III, transcribed with T7; for TNF, 1230 bp cDNA clone (*Eco* R1 ends), cut with *Hind* III, transcribed with T7. The latter two clones were obtained from Dr. A. D.

Weinberg (University of California, San Diego, CA). Human  $\beta$ -actin used as control was from ATCC, clone #78554 cut with *Hind III*, transcribed with T3. All the clones were sequenced from both ends and found to conform to respective cDNA sequences in Genbank.

Ten micrograms of total RNA were fractionated through a 1.2% agarose gel containing 1.1% formaldehyde. The RNAs were transferred to MAGNA NT nylon membranes (Micron Separations Inc. Westboro, MA). The membranes were stained with 0.02% methylene blue and photographed, and the positions of 18S and 28S ribosomal RNA bands and RNA ladder size markers was noted on the membrane and later used for sizing and estimation of total RNA loaded per lane. Prehybridization (approx. 1 hr) and hybridization at 57°C (16 to 24 hr) were carried out in buffers containing 50% formamide and the membranes were washed under high stringency conditions per manufacturer's protocols. The blots were exposed for 6 hr to overnight to Kodak X-AR film at -70°C with Dupont Lightning Plus screens. The bands were quantitated by scanning their densities using ZERO-D scan software program (Stratagene Cloning Systems, La Jolla, CA). Total RNAs per lane were adjusted with the  $\beta$ -actin bands and percentage of change in cytokine RNA levels in mycotoxin- treated mice were calculated using control values set at 1.

IL-2, IL-3, and IFN $\gamma$  and  $\beta$ -actin were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) using Oligo 4.0-software-selected primer sets for each sequence as follows:

IL-2 (611 bp): Sense= 5'-GCGCACCCACTTCAAGCTCC-3'  
Antisense= 5'AGCCCTTGGGGCTTACAAAA-3'

IL-3 (567 bp): Sense= 5'-CCCTTGGAGGACCCAGAAC-3';  
Antisense= 5'-GCCATGAGGAACATT CAGAC-3'.

IFN $\gamma$  (510 bp): Sense= 5'-GTTCTGGGCTTCTCCTCTG-3';  
Antisense= 5'-CGAATCAGCAGCGACTCCTT-3'.

$\beta$ -actin (265 bp): Sense= AACACAGTGTTGTCTGGTGG-3';  
Antisense= 5'-ACGCAGCTCAGTAACAGTCC-3'

For first-strand synthesis, 1  $\mu$ g of lymphocyte total RNA and 0.5  $\mu$ g oligo (dT)<sub>12-18</sub> (GibcoBRL) were mixed in a 20  $\mu$ l total volume reaction containing 50 mM Tris-HCl (pH8.3@RT), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM each dATP, dCTP, dGTP, and dTTP (Promega), and 200U SuperScript II RNase H<sup>-</sup> RT (GibcoBRL) and incubated at 42°C for 50 min. After heating at 70°C for 15 min, the mixture was chilled in ice, and 2 units of RNaseH (Boehringer Mannheim, Indianapolis, IN) were added, followed by a 20-min incubation at 37°C. Twenty microliters of sterile double-distilled water was then added to the cDNA and 2  $\mu$ l of the diluted samples were used for each polymerase chain reaction. The



optimal  $\text{MgCl}_2$  and primer concentrations in a PCR reaction were determined empirically for each cytokine as follows:  $\text{MgCl}_2$ : IL-2 and  $\beta$ -actin= 3mM; IL-3 and  $\text{IFN}\gamma$ = 2mM; Primer concentration: IL-2 and IL-3= 30 pmol;  $\text{IFN}\gamma$ = 20 pmol;  $\beta$ -actin= 10 pmol. Amplification was done in a TemCyclerII thermocycler (Coy Lab. Inc., Ann Arbor, MI) as follows: hot start at 95°C for 30 sec, followed by 25 cycles at 94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min.

The amplification products were run on 2% agarose gels, visualized by ethidium bromide staining, photographed using Polaroid 667 film, and quantitated by scanning the negatives as described for Northern blots.

**Cytokine quantitation.** Supernatants from macrophage cultures activated for 24 hr were used to quantitate IL-1 $\alpha$ , IL-6, and TNF. Supernatants from splenocyte cultures activated for 48 hr were used to quantitate IL-2, IL-3 and  $\text{IFN}\gamma$ . All supernatants were frozen and maintained at -70°C until assayed. All cytokines were quantitated by ELISA, using kits from either Genzyme Diagnostics, Cambridge, MA (IL-1 $\alpha$ , IL-2, IL-6, TNF, and  $\text{IFN}\gamma$ ) or Endogen, Inc., Cambridge, MA (IL-3) per manufacturer's instructions.

**Statistical methods.** Data from these studies were analyzed by one-way analysis of variance (ANOVA) followed by the Fisher PLSD. Statistical calculations utilized Stat view <sup>TM</sup> SE + *Graphics* software from Abacus Concepts, Inc.

## RESULTS

Organ weight determination and cell counts revealed a significant reduction in thymus weight at all doses of T-2 toxin (Table III-1). In contrast, significant higher values were noted in the weight of the spleen at the medium (0.5 mg T-2 toxin/kg body weight) and high (2.5 mg T-2 toxin/kg body weight) doses, and in the liver at the high dose. In addition, a significant increase in white blood cell counts occurred at the medium dose, and a significant reduction of red blood cells at the high dose of T-2 toxin. Kidney weights were unaffected at all doses administered. Thus, it appears that the mycotoxin has an effect in all immunogenic systems measured, the type and extent depending on the dosage. The thymus seems to be particularly sensitive to the effect of the toxin.

The effect of T-2 toxin on IL-2, IL-3, and IFN $\gamma$  gene expression was determined by RT-PCR analysis of total mRNA extracted from splenic lymphocytes of treated and untreated animals, after 12 hr activation with LPS *in vitro* (Figs. III-1-3). IL-2 and IFN $\gamma$  mRNA were significantly increased at the low and high concentrations of the toxin. There was also an increase of these mRNAs at the medium dose level, but the increase was not significant (Figs. III-1 and 3). IL-3 mRNA levels were increased, significantly from the control groups only at the medium dose of the toxin (Fig. III-2). The levels of  $\beta$ -actin mRNA were similar in all the groups.

TABLE III-1

Selected organ weights and blood cell counts of CD 1 mice exposed to  
T-2 toxin for two weeks <sup>a</sup>

T-2 toxin (mg/kg)	Organs (g/100 g body weight)				RBC ( $\times 10^6/\text{mm}^3$ )	WBC ( $\times 10^3/\text{mm}^3$ )
	Liver	Kidney	Spleen	Thymus		
0	5.77 $\pm$ 0.23	1.85 $\pm$ 0.12	0.30 $\pm$ 0.02	0.25 $\pm$ 0.02	8.33 $\pm$ 0.32	5.75 $\pm$ 0.30
0.1	6.05 $\pm$ 0.10	1.65 $\pm$ 0.07	0.39 $\pm$ 0.01	0.19 $\pm$ 0.01*	8.67 $\pm$ 0.13	6.19 $\pm$ 0.50
0.5	6.13 $\pm$ 0.18	1.74 $\pm$ 0.10	0.40 $\pm$ 0.02*	0.20 $\pm$ 0.02*	8.37 $\pm$ 0.23	6.69 $\pm$ 0.55*
2.5	6.60 $\pm$ 0.08*	1.80 $\pm$ 0.10	0.48 $\pm$ 0.06*	0.14 $\pm$ 0.02*	7.16 $\pm$ 0.24*	5.05 $\pm$ 0.38

<sup>a</sup> Values are given as mean  $\pm$  S.E.M., n=5

\* Significantly different from the control value ( $p < 0.05$ )

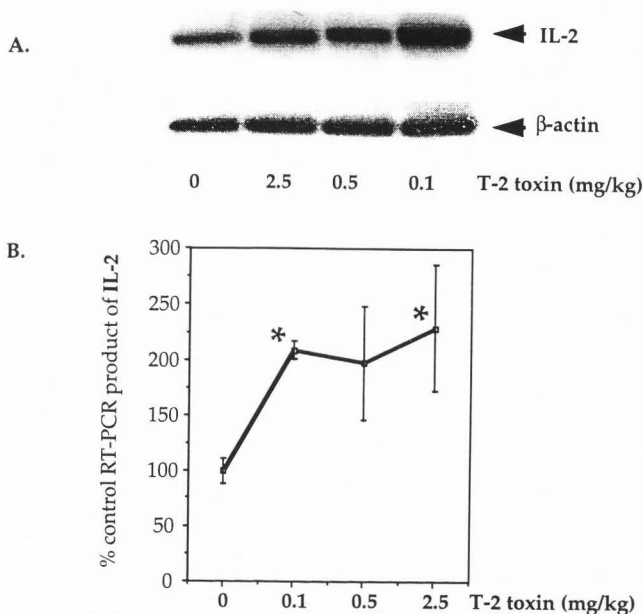


FIG. III-1. Effect of T-2 toxin on the Con A-induced IL-2 expression. Total RNA (1  $\mu$ g) of the Con A-activated ( $5 \mu$ g/ $2 \times 10^6$  cells for 12 hr) splenocytes from control and T-2 toxin-treated mice were used to make cDNA and amplified by RT-PCR using murine IL-2 and murine  $\beta$ -actin primers(A). Band densities from three different animals were measured from the negatives of the ethidium bromide stained 2% agarose gels by Zero D-scan. Values of the total PCR product per lane were measured and equalized using  $\beta$ -actin standards, and the total IL-2 PCR product in the treated groups was calculated as percent of control values(B). Data points were mean  $\pm$  S. E. (n=3). (\*) Differences significant at  $p \leq 0.05$  level.

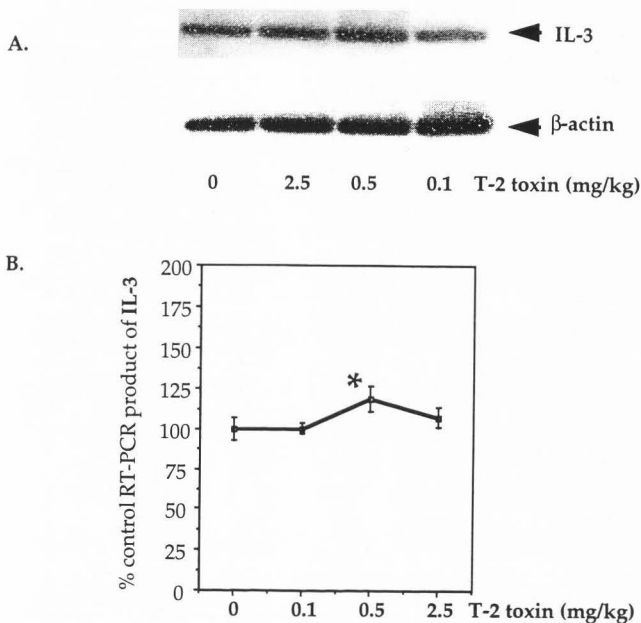


FIG. III-2. Effect of T-2 toxin on the Con A-induced IL-3 expression. Total RNA (1  $\mu$ g) of the Con A-activated (5  $\mu$ g/ $2 \times 10^6$  cells for 12 hr) splenocytes from control and T-2 toxin-treated mice were used to make cDNA and amplified by RT-PCR using murine IL-3 and murine  $\beta$ -actin primers (A). Band densities from three different animals were measured from the negatives of the ethidium bromide stained 2% agarose gels by Zero D-scan. Values of the total PCR product per lane were measured and equalized using  $\beta$ -actin standards, and the total IL-3 PCR product in the treated groups was calculated as percent of control values (B). Data points were mean  $\pm$  S. E. (n=3). (\*) Differences significant at  $p \leq 0.05$  level.

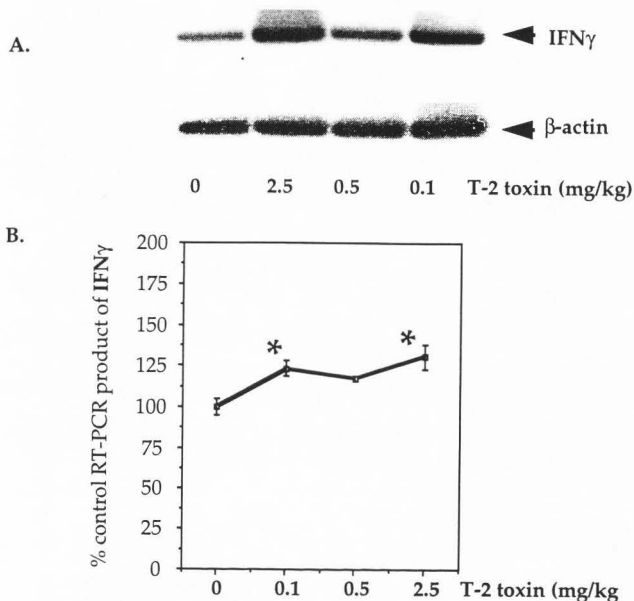
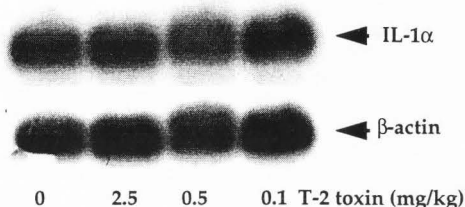


FIG. III-3. Effect of T-2 toxin on the Con A-induced IFN $\gamma$  expression. Total RNA (1  $\mu$ g) of the Con A-activated (5  $\mu$ g/2 $\times$ 10<sup>6</sup> cells for 12 hr) splenocytes from control and T-2 toxin-treated mice were used to make cDNA and amplified by RT-PCR using murine IFN $\gamma$  and murine  $\beta$ -actin primers (A). Band densities from three different animals were measured from the negatives of the ethidium bromide stained 2% agarose gels by Zero D-scan. Values of the total PCR product per lane were measured and equalized using  $\beta$ -actin standards, and the total IFN $\gamma$  PCR product in the treated groups was calculated as percent of control values (B). Data points were mean  $\pm$  S. E. (n=3). (\*) Differences significant at  $p \leq 0.05$  level.

Interleukin-1 $\alpha$ , IL-6, and TNF gene expression in thioglycolate-broth-stimulated, T-2 toxin-treated and untreated animals was determined by Northern blot analysis utilizing the total mRNA extracted from macrophages after 6 hr LPS induction *in vitro* (Figs. III-4-6). There was a significant reduction in the number of peritoneal macrophages obtained from the high-dose group (Table III-2); therefore, not enough total RNA became available for Northern analysis. For this reason, the total RNA extracted from three different animals in the high-dose group was pooled and treated as a single value; thus the results for that group could not be statistically evaluated. The medium and low dose groups show large variations and the values obtained for all three cytokines were not significantly different from the control groups. No marked difference in the levels of  $\beta$ -actin was observed among the groups.

The effect of T-2 toxin on the cytokine protein production was analyzed by ELISA. The supernatants of splenocyte cultures from treated and untreated animals were collected after 48 hr activation with Con A and used to quantitate IL-2, IL-3 and IFN $\gamma$ . Figures III-7 to 9 show that all three cytokine protein levels increased in general at all doses administered; for IL-2 (Fig. III-7) and IFN $\gamma$  (Fig. III-9), these increases were significant at the low dosage only. For IL-3 (Fig. III-8), both the low and medium dosages registered a significant increase in protein secreted into the medium.

A.



B

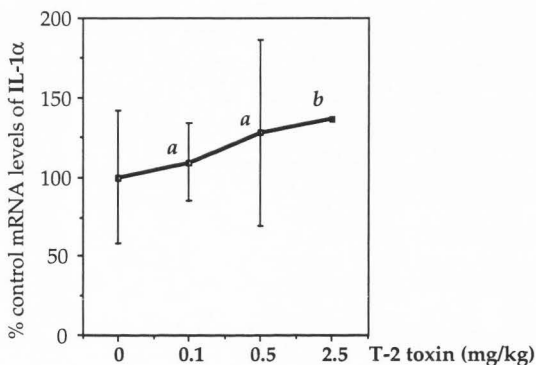


FIG. III-4. Effect of T-2 toxin on the LPS-induced IL-1 $\alpha$  expression. Total RNA (10  $\mu$ g) of the LPS-activated (10  $\mu$ g/1 $\times$ 10<sup>6</sup> cells for 6 hr) peritoneal macrophages from control and T-2 toxin-treated mice were analyzed by Northern blot using murine IL-1 $\alpha$  and human  $\beta$ -actin <sup>32</sup>P-labeled riboprobes in separate experiments(A). Band densities from three different animals were measured in medium and low dose groups and density from high dose group was measured from the mixture of three animals by Zero D-scan. Values of the total RNA per lane were measured and equalized using  $\beta$ -actin standards, and the total IL-1 $\alpha$  mRNA in the treated groups was calculated as percent of control values(B). <sup>a</sup>Data points are mean  $\pm$  S. E. (n=3) from medium and low dose group mice. <sup>b</sup>Single data point was from high dose group mice.



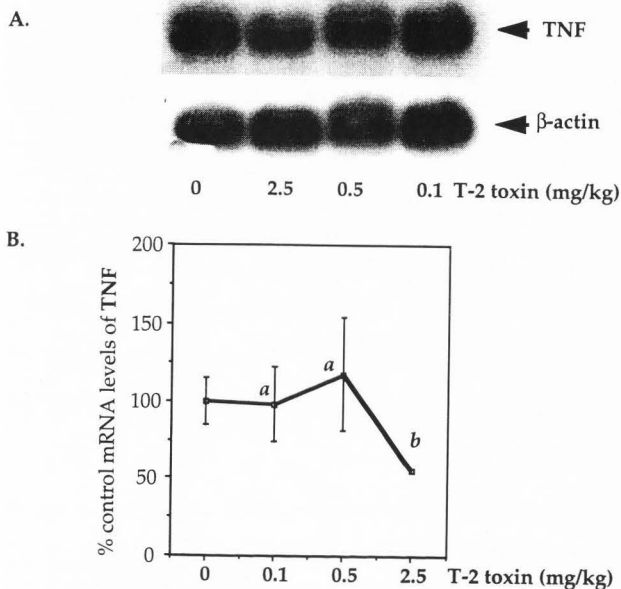


FIG. III-5. Effect of T-2 toxin on the LPS-induced TNF expression. Total RNA (10  $\mu$ g) of the LPS-activated (10  $\mu$ g/ $1 \times 10^6$  cells for 6 hr) peritoneal macrophages from control and T-2 toxin-treated mice were analyzed by Northern blot using murine TNF and human  $\beta$ -actin  $^{32}$ P-labeled riboprobes in separate experiments(A). Band densities from three different animals were measured in medium and low dose groups and density from high dose group was measured from the mixture of three animals by Zero D-scan. Values of the total RNA per lane were measured and equalized using  $\beta$ -actin standards, and the total TNF mRNA in the treated groups was calculated as percent of control values(B). <sup>a</sup>Data points are mean  $\pm$  S. E. (n=3) from medium and low dose group mice. <sup>b</sup>Single data point was from high dose group mice.

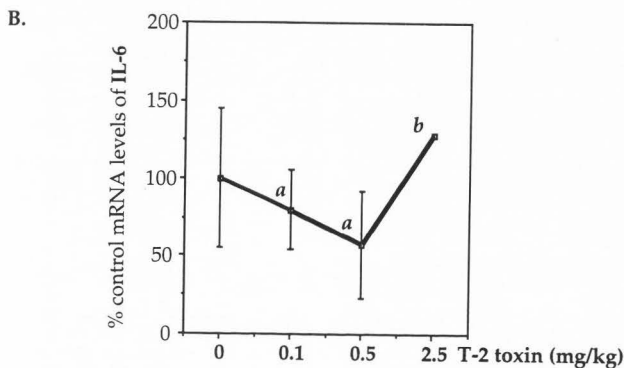
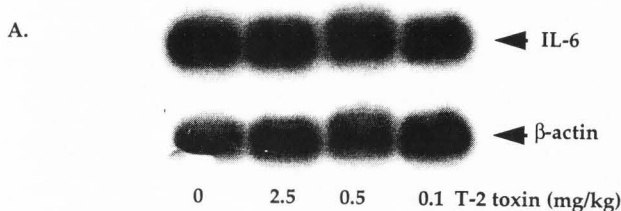


FIG. III-6. Effect of T-2 toxin on the LPS-induced IL-6 expression. Total RNA (10  $\mu$ g) of the LPS-activated (10  $\mu$ g/1x10<sup>6</sup> cells for 6 hr) peritoneal macrophages from control and T-2 toxin-treated mice were analyzed by Northern blot using murine IL-6 and human  $\beta$ -actin <sup>32</sup>P-labeled riboprobes in separate experiments(A). Band densities from three different animals were measured in medium and low dose groups and density from high dose group was measured from the mixture of three animals by Zero D-scan. Values of the total RNA per lane were measured and equalized using  $\beta$ -actin standards, and the total IL-6 mRNA in the treated groups was calculated as percent of control values(B). <sup>a</sup>Data points are mean  $\pm$  S. E. (n=3) from medium and low dose group mice. <sup>b</sup>Single data point was from high dose group mice.

TABLE III-2

Effect of T-2 toxin on the number of peritoneal  
macrophages in CD-1 mice when  
exposed for two weeks <sup>a</sup>

T-2 toxin (mg/kg)	macrophages ( $\times 10^4$ /ml)
0	196.4 $\pm$ 31.23
0.1	228.4 $\pm$ 23.00
0.5	249.0 $\pm$ 9.30
2.5	55.6 $\pm$ 8.70 *

<sup>a</sup> Values are given as mean  $\pm$  S. E. (n=5).

\* Significantly different from the control values ( $p < 0.05$ ).

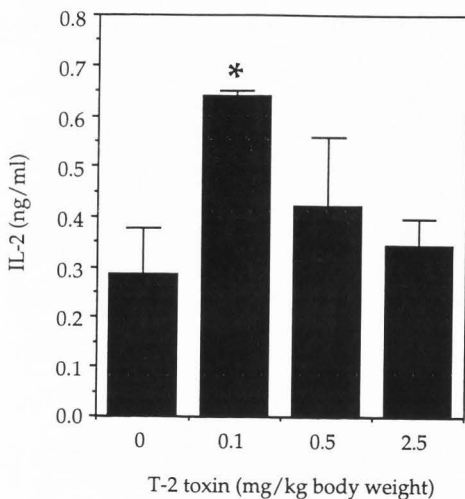


Fig. III-7. Effect of T-2 toxin on IL-2 production. Splenocytes ( $2 \times 10^6$ /ml) from control and T-2 toxin-treated mice were activated with  $5 \mu\text{g}/\text{ml}$  Con A for 48 hrs. Interleukin-2 in culture supernatants was detected by ELISA. Data were mean  $\pm$  S. E. ( $n=4$ ). (\*) Significant difference at 0.05 level between treatment and control groups.

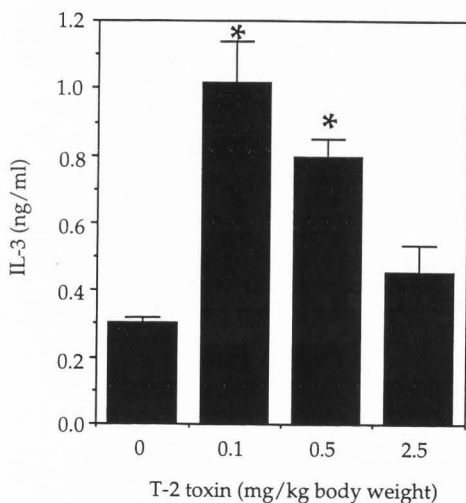


Fig. III-8. Effect of T-2 toxin on IL-3 production. Splenocytes ( $2 \times 10^6$ /ml) from control and T-2 toxin-treated mice were activated with  $5 \mu\text{g/ml}$  Con A for 48 hrs. Interleukin-3 in culture supernatants was detected by ELISA. Data were mean  $\pm$  S. E. ( $n=4$ ). (\*) Significant difference at 0.05 level between treatment and control groups.

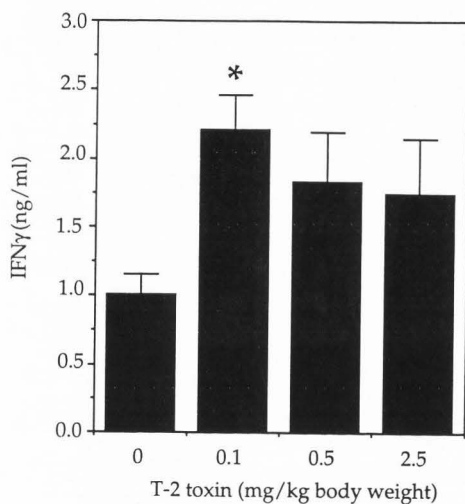


Fig. III-9. Effect of T-2 toxin on IFN $\gamma$  production. Splenocytes ( $2 \times 10^6$ /ml) from control and T-2 toxin-treated mice were activated with 5  $\mu$ g/ml Con A for 48 hrs. Interferon  $\gamma$  in culture supernatants was detected by ELISA. Data were mean  $\pm$  S. E. (n=4). (\*) Significant difference at 0.05 level between treatment and control groups.

At the low dosage, the pattern of protein production for IL-2 and IFN $\gamma$  follows closely that observed for the corresponding mRNA levels (see Figs. III-1, 3). At the medium and high dosages, the higher levels of both mRNA and proteins for these cytokines is maintained, but the protein values do not appear to differ significantly from the controls. IL-3 mRNA and protein levels both increase, and the increase is significant for the protein at the low and medium dosages of the toxin. In general, it seems that T-2 toxin treatment results in increases of both mRNA and protein levels for IL-2, IL-3, and IFN $\gamma$ , depending on the dose. The regulation of gene expression at the transcriptional level is maintained at low doses for IL-2 and IFN $\gamma$ , but not for IL-3, where protein levels secreted into the medium are significantly increased without a corresponding increase in mRNA. At the medium and high doses, the correspondence between mRNA and protein levels is further broken down for all three cytokines, but all register increases in both mRNA and protein levels when compared to the control groups.

Similar ELISA analysis was performed for IL-1 $\alpha$ , IL-6, and TNF using the supernatant from macrophage cultures after LPS induction for 24 hr *in vitro* (Figs. III-10-12). The levels of protein for all three cytokines secreted in the medium are significantly reduced at all dose levels, except for the IL-1 $\alpha$  level found at the medium dose, which, although reduced, does not differ significantly from control values (see Fig. III-10).

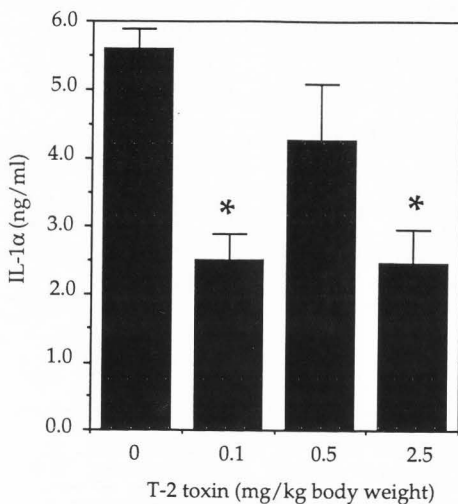


Fig. III-10. Effect of T-2 toxin on IL-1 $\alpha$  production. BTG-stimulated peritoneal macrophages ( $1 \times 10^6$ /ml) from control and T-2 toxin-treated mice were activated with 10  $\mu$ g/ml LPS for 48 hrs. Interleukin-1 $\alpha$  in culture supernatants was detected by ELISA. Data were mean  $\pm$  S. E. ( $n=3$ ). (\*) Significant difference at 0.05 level between treatment and control groups.



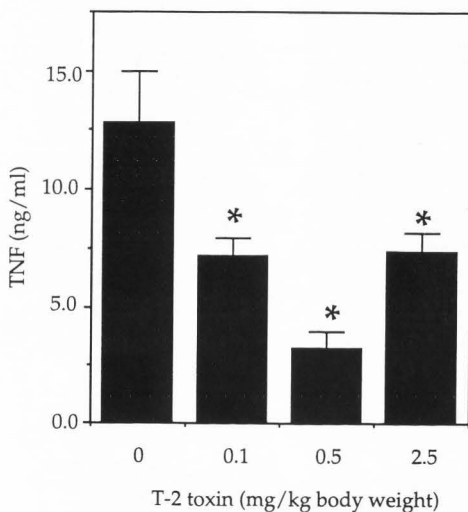


Fig. III-11. Effect of T-2 toxin on TNF production. BTG-stimulated peritoneal macrophages ( $1 \times 10^6$ /ml) from control and T-2 toxin-treated mice were activated with  $10 \mu\text{g/ml}$  LPS for 48 hrs. Tumor necrosis factor in culture supernatants was detected by ELISA. Data were mean  $\pm$  S. E. ( $n=3$ ). (\*) Significant difference at 0.05 level between treatment and control groups.

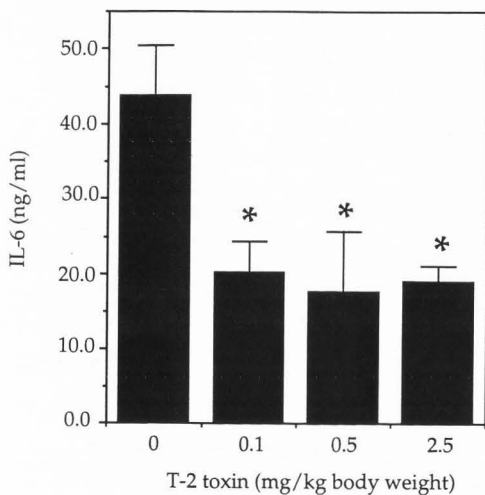


Fig. III-12. Effect of T-2 toxin on IL-6 production. BTG-stimulated peritoneal macrophages ( $1 \times 10^6$ /ml) from control and T-2 toxin-treated mice were activated with  $10 \mu\text{g/ml}$  LPS for 48 hrs. Interleukin-6 in culture supernatants was detected by ELISA. Data were mean  $\pm$  S. E. ( $n=3$ ). (\*) Significant difference at 0.05 level between treatment and control groups.

Thus, T-2 toxin treatment in general seems to have a variable effect on IL-1 $\alpha$ , TNF, and IL-6 mRNA, and to significantly decrease the amount of protein secreted into the medium at all dose levels.

## DISCUSSION

This study measured the effect of T-2 toxin on the genetic expression of certain key cytokines and is based on the assumption that the analysis of the toxin-induced changes in the pattern of gene expression will help to elucidate the molecular mechanisms whereby the toxin exerts its immunosuppressive effects. Low and repeated doses of T-2 toxin were administered *in vivo* and both mRNA and protein levels of cytokines IL-2, IL-3, and IFN $\gamma$  in splenocytes, and cytokines IL-1 $\alpha$ , IL-6, and TNF in macrophages were measured after activation of the purified populations with Con A and LPS, respectively, *in vitro*.

The high dose of T-2 toxin seems to be relatively more toxic; it increased both liver and spleen weights and RBC counts, and decreased the thymus weights significantly. Similar results were observed in other studies (Taylor *et al.*, 1989; Dugyala *et al.*, 1994). In addition, the relative spleen weights and WBC counts of medium-dose-treated animals increased significantly while at the same dose the thymus weights significantly decreased when compared to the control groups; this shows that T-2 toxin affects both the central and peripheral lymphoid organs studied. A decrease of thymus weights at all doses may be due

to cortical depletion (Taylor *et al.*, 1989). Balanced semipurified diet containing T-2 toxin (20 ppm) fed to mice for 6 weeks showed hyperplastic spleens (Hayes *et al.*, 1980). The changes in the spleen were mainly in the red pulp involving proliferation of myeloid components, including erythroid and granulocytic precursors (Taylor *et al.*, 1989). In the present study, the number of brewers thioglycolate broth-activated peritoneal macrophages collected in the high-dose-treated mice was dramatically reduced (4-fold less than the controls). T-2 toxin is more toxic when given by the dietary route than when given by other routes in many species (Ohtsubo and Saito, 1977; Chi *et al.*, 1977; Richard *et al.*, 1978; Weaver *et al.*, 1978; Hayes *et al.*, 1980). In these studies, T-2 toxin consistently caused reduced food consumption, reduced growth rates, and inflammatory lesions around the mouth and in the upper alimentary tract. Similar kinds of symptoms were observed in the present study. In one study (Taylor *et al.*, 1989), the histopathology of male CD-1 mice treated with a high dose (2.5 mg/kg body wt) of T-2 toxin for 2 or 4 weeks included ulcers, partially calcified in the proximal portion of the cardia, with influx of mononuclear infiltration. The reduced number of peritoneal macrophages collected in the high-dose-treated groups in the present study may be due to their accretion in the inflammatory regions. Moreover, T-2 toxin has proven to be cytotoxic to many cell types (Gerberick and Sorenson, 1983; Holt *et al.*, 1988a; Dugyala *et al.*, 1994). T-2 toxin (0.1 - 0.89  $\mu$ M for 20 hr *in vitro*) decreased cell viability, cell number, and viability

index in alveolar macrophages of rats and showed pseudopodia, cellular blebbing, smoothing of membrane processes, and cell lysis when observed by scanning electronmicroscopy (Gerberick and Sorenson, 1983).

There is general induction of IL-2 and IFN $\gamma$  mRNA levels in Con A-activated splenocytes *in vitro* in all doses of T-2 toxin-treated mice; the values are significantly higher than controls in the low and high doses. For IL-3, mRNA levels are significantly higher than controls at the medium dose. It clearly shows that T-2 toxin directly or indirectly induces the mRNA levels of these cytokines either at the transcriptional or post-transcriptional level. The protein levels of all three cytokines are also increased, significantly so for IL-2 and IFN $\gamma$  at low dosage, and for IL-3 at the low and medium dosages. There is a correspondence between mRNA and protein levels for IL-2 and IFN $\gamma$  in the low-dose-treated mice. This is an indication that at low dosage the toxin does not alter the usual transcriptional regulation of IL-2 and IFN $\gamma$  expression. However, in the high-dose-treated mice, protein levels for these cytokines do not correlate with the induced mRNA levels. One possible explanation is that T-2 toxin at the medium and high doses is superinducing mRNA levels by stabilizing the message for these cytokines while reducing the efficiency of translation or post-translational modification of the proteins. The pattern of protein expression for IL-3 does not correspond to the mRNA levels measured at any of the dosages of the toxin;

thus, for this cytokine, the usual transcriptional regulation is also affected by the toxin.

CD4<sup>+</sup> T cells are subdivided into two distinct subsets based on the pattern of lymphocyte secretion; T<sub>H</sub>1 cells produce IL-2 and IFN $\gamma$  (and other cytokines) but not IL-4, IL-5, IL-6, and IL-10. These cells are involved in cell-mediated immune responses. On the other hand, T<sub>H</sub>2 cells produce IL-4, IL-5, IL-6, and IL-10, but not IL-2 and IFN $\gamma$ ; they are involved in antibody-mediated immune responses. IL-3 is produced by both types of subsets (Mosmann and Coffman, 1989). This division of labor among T-helper cells determines whether humoral or cell-mediated immunity will predominate following their first encounter with antigen. The high levels of IL-2 and IFN $\gamma$  measured after the low-dose treatment is an indication that, at that dosage, T-2 toxin may increase cell-mediated immunity. This does not necessarily translate into an enhanced immune response since, in order to be effective, these cytokines must interact with their target cells and these, in turn, may be impaired by other toxin-induced alterations, such as those found in the macrophages to be discussed below.

It is known that these cytokines are tightly regulated both transcriptionally and post transcriptionally. Interleukin-2, IL-3, and IFN $\gamma$  cytokine genes share a consensus element, TTATTTAT, located in the 3' untranslated region, which is believed to confer instability to the transcripts resulting in their rapid degradation (Campbell *et al.*, 1985; Caput *et al.*, 1986;

Shaw and Karmen, 1986). Cyclohexamide (CHX), a protein synthesis inhibitor, has been shown to superinduce IL-2 and IFN $\gamma$  mRNA. The superinduction is presumably due to stabilization of the mRNA because of CHX inhibition of RNase synthesis responsible for the specific degradation of the unstable transcripts (Cockfield and Ramassar, 1991; Zubiaga *et al.*, 1991; Cockfield *et al.*, 1993). The observation that T-2 toxin increases mRNA levels of IL-2, IL-3 and IFN $\gamma$  and that it, in general, increases the levels of secreted protein in the medium suggests that its action resembles that of CHX. Vomitoxin, another trichothecene, produced by *Fusarium graminearum*, also superinduced the production of IL-2, IL-4, IL-5, and IL-6 mRNA and protein levels in EL4.IL-2 thymoma cell line *in vitro* (Dong *et al.*, 1994). A 4-fold increase in IL-2 levels was observed in Con A-activated splenocytes *in vitro* from mice dosed for four consecutive doses of 2 mg/kg T-2 toxin (Holt *et al.*, 1988b). Enhanced resistance to such infections as *Listeria monocytogens* due to preinoculation treatment with T-2 toxin in some studies (Corrier and Ziprin, 1986; Corrier *et al.*, 1987) can be partially explained as being due either to the capacity of T-2 toxin to induce IL-2, IFN $\gamma$ , IL-3 and increase cell mediated immunity, or to an increase of phagocytic activity of macrophages. However, T-2 toxin immunotoxicity definitely depends on its dose and duration of exposure in the mice. T-2 toxin treatment (0-2.5 mg/kg) every third day in CD-1 mice for 2 weeks increased PHA-induced stimulation *in vitro* in splenocytes in low-dose-treatment groups and suppression

in high-dose-treatment groups in splenic lymphocytes, and no significant differences were observed when the treatment extended to 4 weeks (Taylor *et al.*, 1985). T-2 toxin is also known to decrease resistance to infections (Kanai and Kondo, 1984; Corrier and Ziprin, 1986) and cause a decrease in cell and humoral-mediated immunity (Rosenstein *et al.*, 1979; Taylor *et al.*, 1985). Activation or no effect of the IL-2, IFN $\gamma$ , and IL-3 gene expression due to T-2 toxin exposure in the present study shows that T-2 toxin-induced immunosuppression may not be due to alteration of these cytokines, but some other mechanism. Thymic atrophy, depletion of T lymphocytes, and prolymphoid population in fetal thymocytes (Hayes *et al.*, 1980; Hunter *et al.*, 1985; Taylor *et al.*, 1989; Holladay *et al.*, 1993; Holladay *et al.*, 1995) due to the T-2 toxin exposure in mice shows that T-2 toxin is specifically lymphocytolytic. Even though elevated levels of cytokines are observed, lack of enough responding cells to the specific cytokines may cause immunosuppression.

T-2 toxin seems to have a profound effect on the macrophage-produced cytokines when activated with LPS *in vitro*. As pointed out earlier in the discussion, there was a reduction in the number of macrophages in the high-dose-treated groups, which may be due to the concentration of macrophages in the inflammatory sites, rather than their accumulation in the peritoneal fluid. The total mRNA was pooled and analyzed as a single group. This group of animals showed a slight decrease in the TNF mRNA when compared to the controls even



though the decrease cannot be evaluated statistically. In general, T-2 toxin does not seem to affect the mRNA levels of IL-1 $\alpha$ , IL-6, and TNF but inhibits the secreted protein levels in all doses (IL-1 $\alpha$  protein is not significantly decreased in the medium dose group). The data show that in the macrophage, the toxin regulates the expression of the cytokines measured mainly at the translational or post-translational level.

T-2 toxin is known to be a protein synthesis inhibitor in immune cells (Gyongyossy-Issa and Khachatourians, 1984; Rosenstein and Lafarge-Frayssinet, 1983). This inhibition may be due to direct action of the toxin on the protein synthesizing machinery of the cell, or it could be acting indirectly via some other pathway, i.e., the stress-related response involving the HPA axis. Evidence for a direct effect of the toxin on protein synthesis was obtained by Cundliffe *et al.* (1974), who showed that T-2 toxin impairs polypeptide chain initiation by causing disaggregation of the polyribosomes. It is also known that trichothecenes, including T-2 toxin, bind to ribosomes and thereby inhibit peptidyl transferase and block initiation of polypeptide synthesis (Melmed and Yagen, 1985). Indirect action of T-2 toxin on the macrophage cytokine production is also substantiated. Endotoxemia is observed 24 hr after male CD-1 mice received a single oral dose of T-2 toxin (2.5-20 mg/kg) coinciding with the increase of corticosterone levels (Taylor *et al.*, 1989). Endotoxemia in male Swiss mice following treatment with T-2 toxin (4 mg/kg) was found to be due to increased absorption of endotoxin,

an outer membrane component of gram-negative bacteria, from the gastrointestinal tract (Taylor *et al.*, 1991). Even though they observed the increased levels of endotoxin and corticosterone levels in the higher concentration of the toxin, it is possible that these levels may also be increased in all doses used in this study. Corticosterone is known to selectively deplete the thymic cortical cells in mice (Carroll *et al.*, 1969). This correlates with the significant decrease in thymic weights in all doses observed in this study, indicating that there is a hormonal imbalance due to a stress response associated with systemic endotoxemia.

Endotoxin tolerance is the phenomenon developed by the host to survive and elicit controlled inflammatory responses due to a localized stimulus, resulting in repeated insult with the endotoxin (Zuckerman *et al.*, 1991). It is possible that in the present study, peritoneal macrophages became tolerant due to endotoxemia *in vivo*, thus becoming unable to secrete the cytokines upon further activation with LPS *in vitro*. Regulation of TNF expression in peritoneal macrophages that were refractory following repeated *in vitro* exposure to LPS has been demonstrated to occur at a post-translational level (Zuckerman *et al.*, 1989). Involvement of glucocorticoid hormones in this process has been shown (Beutler *et al.*, 1986). IL-1 translation is also suppressed in LPS-activated U937 cells *in vitro* when exogenous prostaglandins (PGE<sub>2</sub>) were added, via increase of cellular cAMP levels (Knudsen *et al.*, 1986). Endotoxin treatment is known to

increase PGE<sub>2</sub> synthesis in monocytes (West *et al.*, 1993). Corticotropin releasing factor induces not only the release of adrenocorticotrophic hormone by the pituitary, which induces corticosterone via the HPA axis in adrenals, but also the release of catecholamines such as norepinephrine from adrenal medulla (Fuchs and Sanders, 1994). Norepinephrine seems to increase cAMP levels (Krammer, 1988). It is possible that the 3' untranslated sequence (TTATTAT), which is conserved in pro-inflammatory cytokines (Brown and Beutler, 1990), may be regulated translationally through a cAMP-dependent signal transduction mechanism.

In conclusion, T-2 toxin appears to affect cytokine production by lymphocytes and macrophages differentially. The lymphocyte-produced cytokine mRNA and protein levels are generally increased, and the macrophage-produced cytokines are suppressed at the protein levels. The decrease in protein levels observed in the macrophage may be due to a direct effect of the toxin on the protein synthetic machinery of the cells, or an indirect response through a stress-related mechanism. The immunosuppression caused by the toxin may be due to the impairment of macrophage functions, or to the fact that, even in the presence of increased amounts of cytokine proteins (IL-2, IFN $\gamma$ , or IL-3), cytolytic action or impairment of membrane function by the toxin on effector cells, *c.f.*, macrophages, cytotoxic/helper T cells, NK cells, etc., obviates the production of a full immune response.

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## CHAPTER IV

## SUMMARY

Mycotoxins are secondary fungal metabolites; they are ubiquitous in the environment and have a wide range of toxic effects, including immunotoxicity to humans as well as domestic and laboratory animals. Aflatoxin B<sub>1</sub> is produced by several strains of *aspergillus* species and T-2 toxin is produced by the *fusarium* species. Immunotoxicity of both toxins is mediated by their influence upon non-specific immune processes such as adherence, phagocytosis, and chemotaxis by macrophages and other heterophils. Cell- and humoral-mediated immunity are modulated by affecting mainly T-helper cell functions. This would inhibit delayed-type hypersensitivity; cytotoxicity by NK, CTL, and macrophages; and T-dependent antibody production. Interestingly, many of the above mentioned immune functions are controlled by growth factors collectively known as cytokines. Little information is known about the modulation of cytokine gene expression by the mycotoxins, specifically, whether they influence their transcriptional and/or translational regulation.

The present study investigated the molecular events involved in AFB<sub>1</sub>- and T-2 toxin-induced immunotoxicity in male CD-1 mice by measuring gene expression of cytokines produced by peritoneal macrophages (IL-1 $\alpha$ , TNF, and IL-6) and lymphocytes (IL-2, IL-3, and IFN $\gamma$ ). Messenger RNA and protein levels

were measured in LPS-activated macrophages and Con A-activated lymphocytes *in vitro*: Cytokine-specific mRNAs were identified and quantified by Northern blot/RT-PCR techniques, and protein levels were measured by sandwich ELISA. Male CD-1 mice received either 0 (control), 0.03 (low dose), 0.145 (medium dose), and 0.7 (high dose) mg/kg body weight of AFB<sub>1</sub>, or 0 (control), 0.1 (low dose), 0.5 (medium dose), and 2.5 (high dose) mg/kg body weight of T-2 toxin, by oral gavage every other day for 2 weeks (seven doses).

The results clearly show that the toxicology and alteration of cytokine expression of AFB<sub>1</sub> and T-2 toxin are entirely different. Aflatoxin B<sub>1</sub> has no effect on the weights of the organs selected but does have a significant increase in the WBC count in the low-dose-treated group. T-2 toxin significantly suppressed the thymus weights in all groups, and increased the spleen weights in medium- and high-dose-treated groups. A significant increase of liver weight, and an increase of RBC counts in high dose and WBC counts in medium dose show that T-2 toxin affects some of the vital organs of the body. A 4-fold decrease in the total number of macrophages collected in high-dose-treated T-2 toxin group confirms that T-2 toxin is toxic in the high-dose-treated mice.

Con A-induced production of cytokine profile from AFB<sub>1</sub>-treated mice splenocytes is different from the cytokines produced from T-2 toxin-treated mice. A significant decrease of IL-2 mRNA and a pattern of slight decrease in IFN $\gamma$  and IL-3 mRNA in the low-dose-treated AFB<sub>1</sub> groups are observed. Protein levels of

these cytokines followed the pattern of mRNA of the respective cytokine; this shows that there is no decoupling of mRNA and protein synthesis. These results show that AFB<sub>1</sub> did not have much effect on the expression of these cytokines. A slight decrease of these cytokines in the low dose-treated group may be due to the activation of inhibitory factors for T<sub>H</sub>1-producing cytokines.

T-2 toxin, on the other hand, induced the IL-2, IFN $\gamma$ , and IL-3 mRNA expression in *in vitro* Con A-activated splenocytes. Interleukin-2 and IFN $\gamma$  mRNA were induced significantly in high- and low-dose-treated mice, and IL-3 mRNA is induced in medium-dose-treated mice. Protein levels of IL-2 and IFN $\gamma$  followed the mRNA levels in the low dose but not in the high dose. These results suggest that T-2 toxin is either increasing the transcription or stabilization of mRNA in the low- and high-dose-treated mice and at the same time interfering with the protein synthesis or secretion of these cytokines in the high-dose-treated-mice. Interleukin-3 protein levels are significantly high in the medium and low dose even though there is no significant increase in the mRNA levels in the low-dose-treated mice, suggesting that T-2 toxin also increases the translational or post-translational efficiency. These results also reveal that cytokines are modulated differentially by these toxins.

Lipopolysaccharide-induced IL-1 $\alpha$ , TNF, and IL-6 gene expression in peritoneal macrophages is also modulated differently by both toxins in exposed mice. Messenger RNA expression of both IL-1 $\alpha$  and TNF was suppressed

significantly in high dose and increased significantly in low and medium dose, respectively, in AFB<sub>1</sub>-treated mice. Protein of both cytokines decreased significantly in accordance with the mRNA. Interleukin-1 $\alpha$  protein is strongly suppressed also in low- and medium-dose-treated mice. Interleukin-6 mRNA is increased significantly in the medium dose group and there is a general decrease the protein in all doses, which is significant in high-dose-treated groups. Aflatoxin B<sub>1</sub> seems to modulate both mRNA and protein levels differently in all groups. Suppression or activation of mRNA levels by AFB<sub>1</sub> may be due to modulation of factors responsible for transcription or mRNA stability. Strong suppression of IL-1 $\alpha$  protein in all groups of treated mice is a very interesting result because of the unique mechanism of processing and secretion of IL-1 $\alpha$  when compared to the other pro-inflammatory cytokines.

There are no significant differences in the mRNA levels of IL-1 $\alpha$ , TNF, and IL-6 in LPS-activated peritoneal macrophages of low- and medium-dose-treated T-2 toxin mice. Significant levels could not be assessed statistically in the high-dose-treated group because pooled RNA from three animals is used as a single group due to lack of sufficient RNA. However, a slight decrease in TNF mRNA was observed with no difference in the other two cytokines in the high-dose-treated mice. Protein levels in all cytokines are suppressed significantly (except IL-1 $\alpha$  in medium dose) in all treated doses; this shows that T-2 toxin has



more of an effect at the translational or post-translational level in a nonspecific manner.

In short, AFB<sub>1</sub> and T-2 toxin differ in the way they modulate cytokine expression in CD-1 mice. Based on these results and others, AFB<sub>1</sub> may modulate the cell-mediated responses, inhibiting the macrophage-producing cytokines and other functions, thereby stopping the signals to T-helper cells. T-2-toxin-induced immunosuppression may also be due to the inhibition of cytokines produced by the macrophages, but lack of effector cells to respond to the cytokines (IL-2, IFN- $\gamma$ , and IL-3) is also an important factor in the mechanism of immunosuppression of T-2 toxin. Figure IV-1 presents a summary based on the present data of the possible mechanisms through which AFB<sub>1</sub> and T-2 toxin modulate cytokine gene expression.

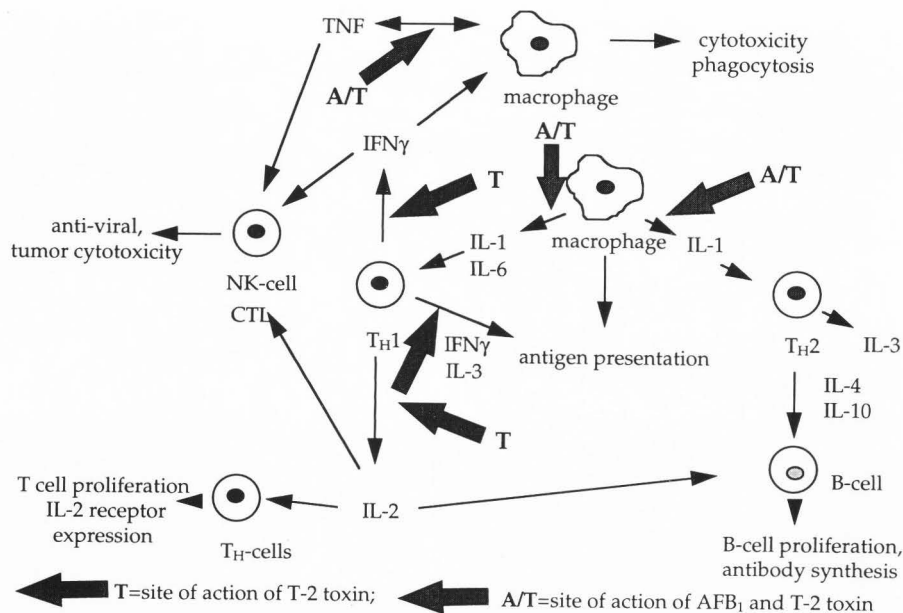


Fig. IV-1. The proposed mechanism of action of AFB<sub>1</sub>/T-2 toxin on cytokine communication network in male CD-1 mice.

## CURRICULUM VITAE

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**CAREER  
OBJECTIVES**

To obtain a post-doctoral fellowship in the area of toxicology involving elucidation of molecular mechanisms of toxicity.

**RESEARCH  
SKILLS**

- Genetic engineering, isolation and characterization of acids.
- Reverse-Transcription Polymerase Chain Reaction (RT-PCR).
- cDNA cloning and sequencing, electroporation.
- *in vivo* and *in vitro* evaluation of general and immunotoxicity.
- Handling of laboratory animals (mice), experience in oral gavage, i.p., i.v. injection of toxic chemicals/drugs.
- Cell culture.
- Sandwich ELISA and cytokine bioassays, CMI.
- Laboratory handling of toxic and radioactive materials.
- Knowledge in GCG, oligo 4.0, amplify 1.2, DNA strider, zeroD scan, microsoft office.
- Training and supervision of students and technicians in the laboratory.

**RESEARCH  
EXPERIENCE**

- Immunotoxicity of xenobiotics (1991-1995).

The main goal of these studies is to find out the immuno-modulatory effects and possible mechanisms of Immunotoxicity of aflatoxin B<sub>1</sub> and T-2 toxin in male CD-1 mice. Effects on various cytokines at mRNA and protein levels were studied assuming the hypothesis that these toxins may produce their immunotoxic effects via interference with the synthesis of different cytokines in T lymphocytes (IL-2, IL-3 and IFN $\gamma$ ) and macrophages (IL-1 $\alpha$ , IL-6 and TNF).

Myelotoxic effects of low and repeated doses of mycotoxin were studied *in vivo* in male CD-1 mice granulocyte-macrophage (GM) progenitor cells. *In vitro* effects on normal GM-progenitor cells were also investigated.

- Over four years of experience in the area of molecular mechanisms of toxicity (1991-1995).

The molecular mechanisms of retinoic acid (vitamin A-derivative)-induced teratogenesis in hamster embryo were studied. Also involved in the retinoic acid receptors (RAR $\alpha$ ,  $\beta$ ,  $\gamma$ ) sequencing and their regulation in hamster embryos.

- Junior Research Fellow at immunobiology lab, Osmania University, Hyderabad, India (1987-1990).

The projects involved were on immunomodulatory effects of dapsone and DDT in mice.

#### TEACHING EXPERIENCE

- Over three years of experience in teaching, undergraduate biochemistry and immunology (1984-1987).

Helped teach laboratory techniques, conducted and evaluated examinations.

#### EDUCATION

**Ph.D. Toxicology** (September, 1991-September, 1995)  
Utah State University, Logan, Utah. Dissertation title:  
Alteration of key cytokine levels by alfatoxin B<sub>1</sub> and T-2  
toxin in male CD-1 mice.

**M.S. Zoology** (1984-1987)  
Kakatiya University, Warangal, India.

**B.S. Biology** (1981-1984)  
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#### SELECTED COURSES

Environmental and Industrial Toxicology, Principles of Toxicology, General Pharmacology, Immunology, Molecular Biology, Biochemistry, Cell Biology, Animal Pathobiology, Physiology, Biostatistics.

#### AWARDS AND HONORS

Graduate Research Assistantship, Utah State University (1991-95).

Junior Research Fellowship, University Grants Commission, India (1987-1990).

National Merit Scholarship, Kakatiya University, India  
(1984-1987).

Student member, Mountain West Society of Toxicology, 1992

## ORGANIZATIONS

**PUBLICATIONS** **Dugyala, R.R.** (1995). Alteration of key cytokine levels by aflatoxin B<sub>1</sub> and T-2 toxin in male CD-1 mice. Ph.D. dissertation, Utah State University, Logan, Utah.

**Dugyala, R.R.**, Colon-Teicher, L., Warren, R.P., and Sharma, R.P. (1995). Alteration of key cytokines produced by mitogen-activated peritoneal macrophages and splenocytes in aflatoxin B<sub>1</sub>-treated male CD-1 mice. In preparation.

**Dugyala, R.R.**, Colon-Teicher, L., Warren, R.P., and Sharma, R.P. (1995). Alteration of key cytokines produced by mitogen-activated peritoneal macrophages and splenocytes in T-2 toxin-treated male CD-1 mice. In preparation.

Colon-Teicher, L., **Dugyala, R.R.**, and Sharma, R.P. (1995). Regulation of retinoic acid receptors  $\alpha$ ,  $\beta$ ,  $\gamma$  mRNA in hamster embryos and effect of RA. Submitted: *Mol. and Cell. Endocrinol.*

**Dugyala, R.R.**, Kim, Y-W., and Sharma, R.P. (1994). Effects of aflatoxin B<sub>1</sub> and T-2 toxin on the granulocyte-macrophage progenitor cells in mouse bone marrow cultures. *Immunopharmacology* 27, 57-65.

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## ABSTRACTS

**Dugyala, R.R.**, Kim, Y-W., and Sharma, R.P. (1993). Effects of aflatoxin B<sub>1</sub> and T-2 toxin on the granulocyte-macrophage progenitor cells in mouse bone marrow cultures. Presented at Society of Toxicology, 1993, New Orleans, LA.

**Dugyala, R.R.**, Kim, Y-W., and Sharma, R.P. (1992). Effects of aflatoxin B<sub>1</sub> on the granulocyte-macrophage progenitor cells in mouse bone marrow cultures. Presented at Mountain West Society of Toxicology, 1992 annual meeting, Utah State University, Logan, Utah.